Industrial Production of γ-Linolenic Acid by Filamentous Fungi(Dissertation_全文)

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ABBREVATIONS

16:0	palmitic acid
16:1	palmitoleic acid
18:0	stearic acid
18:1	oleic acid
18:2	linoleic acid
18:3	linolenic acid
C/N ratio	ratio of carbon to nitrogen atom weight
CF	crystallized fraction
D	dilution rate
DC	dry cell weight
DG	diacylglycerol
DGLA	dihomo- γ -linolenic acid (*cis*-8,*cis*-11,*cis*-14 eicosatrienoic acid)
DU	digree of unsaturation
FAME	fatty acid methyl esters
FFA	free fatty acid
FS	free sterol
G_I	inlet glucose concentration
G_S	glucose concentration at steady-state
GC	gas chromatography
GLA	γ -linolenic acid (*cis*-6,*cis*-9,*cis*-12 octadecatrienoic acid)
HPLC	high-performance liquid chromatography
LF	liquid fraction
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine

MG	monoacylglycerol
NL	neutral lipids
NL/DC	neutral lipid content in dry cell
P_C	productivity of the cell mass
P_L	lipid productivity
p-GL	posphorus glycolipid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	polar lipids
PL/DC	polar lipid content in dry cell
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
SE	sterol ester
TCN	theoretical carbon number
TG	triacylglycerol
TL	total lipid production
TL/DC	lipid content in dry cell
TLC	thin layer chromatography
TPL	total polar lipids detected by HPLC
UFA	total unsaturated fatty acids
Y_f	yield of fraction
Y_{GLA}	yield of GLA
η_{GLA}	separation efficiency for GLA
η_x	separation efficiency for x

INTRODUCTION

Gamma-linolenic acid (GLA), *cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid, or 18:3(n-6), is an intermediate in the metabolic pathway of (n-6) essential fatty acid from linoleic acid into arachidonic acid, the precursor of 2-series icosanoids (prostaglandins (PG), thromboxanes, and leukotrienes), which play vital roles in the human body. Further, GLA is the direct precursor of dihomo- γ -linolenic acid (DGLA), 20:3(n-6), the precursor of anti-inflammatory 1-series icosanoids[1] as shown in **Figure 1**. The effectiveness of GLA supplementation derives from the facts that activity of Δ 6 desaturase, that converts linoleic acid into GLA, has been found to fall rapidly in the testes and more slowly in the liver in aging animals. Other factors which inhibit Δ 6 desaturase are diabetes, alcohl, radiation, high carbohydrate diet, low protein diet. Delta-6-desaturasion is the rate-limiting step in the metabolic pathway of (n-6) essential fatty acid even in the young animals, which causes deviation of icosanoids profile, especially in the case of symptoms above mentioned[2-5]. Dietary GLA was also shown to cause stimulation of Δ 6 desaturase both in (n-6) and (n-3) essential fatty acids[6,7]. This stimmulation as well as supplemented GLA itself cause higher level of DGLA, which has anti-thrombogenic, anti-atherosclerotic including hypocholesterolic, anti-inflammatory[8-10], and other effects[6, 11,12]. These are the rationale that GLA will be used as functional foods and pharmaceutical products. On the other hand, it is not easy for us to take in some level of GLA from conventional foods which in many case contains little GLA. Gamma-linolenic acid or lipid containing GLA is obtained conventionally from several plant seeds, such as those of evening primrose (*Oenothera biennis L.*)[13], which contains about

8% of GLA and is available commercially as a health food.

The author has studied the production of fats and oils by fungi, in order to develop new sources of fats and oils. In Japan, the major sources of fats and oils, such as soybean, palm, rape, sunflower, tarrow, lard, groundnut, cotton, coconut, are mostly imported from other countries. Therefore, research on microbial production of oils and fats would be indispensable in terms of national security and global resource preservation. Many studies on lipid production with oleaginous yeasts have been performed since the 1940s[14-18]. However, not many researches on lipid production by fungi have been carried out[19], which was caused by the difficulty of realizing cultures at higher cell concentration and lipid productivity compared with yeasts. In 1965, Shaw[20] reported the existence of GLA only in the Phycomycetes (including Oomycetes and Zygomycetes), but not in other classes such as the Ascomycetes and Basidiomycetes. Tyrrell[21] also reported that some Oomycetes and Zygomycetes contain DGLA and arachidonic acid.

Recently, research on the microbiological production of GLA and other polyunsaturated fatty acids (PUFAs), difficult to obtain from plant or animal oils, has been actively performed, based on those early studies. However, almost all these researches have not been extended to practical production, because of their low PUFA contents as well as the low productivities. Shimizu *et al.* found *Mortierella alpina* among Zygomycetes fungi, isolated from soil, produced a considerable amount of arachidonic acid[22,23]. They also reported the production of DGLA, icosapentaenoic acid and some other PUFAs by the strain and its mutant strains[24-26].

The author has developed a method for the production of GLA or a

lipid containing GLA by the culture of a particular fungal strain in a liquid culture medium with markedly increased efficiency, which was eventually advanced to practical production.

In chapter I, the author describes investigation of cell growth, lipid production, lipid distribution and lipid productivity among members of the order Mucorales in the Zygomycetes. This chapter deals with the search for a fungus exhibiting high productivity of lipids or GLA.

In chapter II, the author describes investigation of the influence of the cultural conditions on cell growth, lipid composition and lipid accumulation, in order to increase in the lipid production by *Mortierella isabellina*, the selected strain in chapter I.

In chapter III, the author describes the production of GLA by the genus *Mortierella* resulting in a high cell mass and a high concentration of GLA-containing lipids.

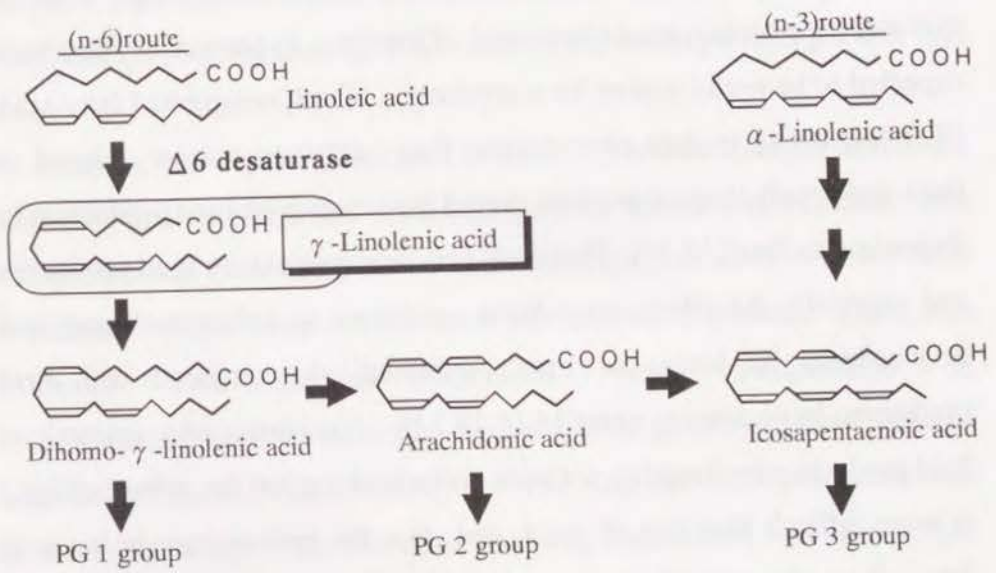


Figure 1. Pathways for the biosynthesis of prostaglandin (PG) groups.

CHAPTER I

Lipid Production and γ-Linolenic Acid Content of Genus *Mortierella*

Section 1 Lipid composition of 6 species of order Mucorales in class Zygomycetes

Many microorganisms have been characterized with respect to their cellular fatty acid composition, mainly from a taxonomic view point[27,28]. Fungus class Zygomycetes is characteristic of having γ-linolenic acid (*cis*-6,*cis*-9,*cis*-12 octadecatrienoic acid, GLA) for a fatty acid instead of α-linolenic acid (*cis*-9,*cis*-12,*cis*-15 octadecatrienoic acid), therefore, many researches were done regarding its lipid. In 1965, Shaw[20] proved the existence of GLA in the Pycomycetes (including Oomycetes and Zygomycetes) only, but not in other classes such as the Ascomycetes and Basidiomycetes. Tyrrell[29] also described that Zygomycetes fungi of *Entomophera* contain arachidonic acid. Therefore, Zygomycetes fungi were expected to be useful source for a production of polyunsaturated fatty acids (PUFAs), owing to their characteristic fatty acid composition. Based on these early study, several workers started the research on lipid production by Zygomycetes fungi[30,31]. However, very few research on lipid production and especially the effects on cultural conditions on cell growth and lipid accumulation by fungi has been performed[19], compared with lipid production by oleaginous yeast[14,16-18,32]. The reason why research on lipid production by fungi has not been performed are that the culture of fungi is more difficult than that of yeast, and that the lipid content is lower in fungi. Recently, research on microbial production of some PUFAs has been actively performed, based on these early studies. However, almost of these

researches have not been extended to practical production, because of the low PUFA content as well as low productivity.

This section deals with the investigation on fatty acid composition, lipid distribution and lipid productivity of 6 species, 11 strains of order Mucorales in Zygomycetes.

Materials and Methods

Microorganisms. Order Mucorales 6 species (11 strains), *Abisidia corymbifere, Choanephora circinans, Mortierella isabellina, Mucor ambiguus, Rizopus oryzae* and *Syncephalastrum racemosum* were obtained from the Culture Collection of the Institute of Fermentation, Osaka.

Media and culture conditions. The microorganisms were grown in 500 mL erlenmeyer flasks, containing 200 mL medium. The flasks were incubated at 20 or 30℃ under stationary conditions. Liquid culture medium consisted of 30 g glucose, 3.0 g KH_2PO_4, 3.0 g NH_4NO_3, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.2 g malt extract, 0.2 g yeast extract, 10 mg $FeSO_4 \cdot 7H_2O$, 1.2 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$ and 1.0 mg $ZnSO_4 \cdot 7H_2O$ per liter (pH 4.6).

Analysis of cell growth and lipid content. Mycelia were harvested at a stationary growth phase and separated from the medium by filtration. The cell growth was determined by dry cell weight (DC) in an oven at 110℃ to a constant weight using a portion of the filtrated mycelium. Lipid was extracted from filtrated mycelium with homogenation in chloroform/methanol solution (2:1 by vol.) with glass beads and purified according to the methods of Folch[33]. The total lipid (TL) production was measured by weight. The extracted lipid was fractionated into neutral lipid (NL) and polar lipid (PL) by silicic acid chromatography, as described by Vance and Sweeley[34].

Analysis of lipid. Distribution of NL and PL were analyzed by thin layer chromatography (TLC) according to the method of Suzuki *et.al.*[35,36] with a slight modification using silica gel-precoated TLC plate (Merck 5721, 20×20 cm, 0.25 mm thickness). The solvent system used for NL analysis was (a) benzene/diethyl ether/ethanol/28% ammonia water (50:40:2:0.5 by vol.) and (b) *n*-hexane/diethyl ether (94:6 by vol.). The plates were, first, developed from origin to a level of 12 cm high using solvent (a), after drying, secondly developed to a level of 17 cm high using solvent (b). The solvent system used for PL analysis was chloroform/ acetone/methanol/acetic acid water (50:20:10:10:5 by vol.)[37]. The plates were then sprayed with 13% H_2SO_4 and 2% $CuSO_4 \cdot 5H_2O$ solution, and heated in an oven at 140℃ for 10 min. The lipids were identified with authentic standards or with some specific coloring reagents such as ninhydrin for free amino groups, molybdic-blue for phospholipids, Dragendorff reagent for choline and 1-naphthol for glycolipids. Quantitative analysis of NL distribution was performed by a densitometric method, using a TLC scanner (Shimadzu, Model CS-910) with a zigzag scanning mode. Each fraction of phospholipids separated from PL on the TLC plate was scraped from the plate, and the scraped sample was then analyzed by the method of Bartlett[38].

Fatty acids were analyzed as methyl esters prepared by using a 7% BF_3/methanol solution after saponification with 0.5 N methanolic NaOH. Then the fatty acid methyl esters were analyzed with a gas chromatograph (GC) equipped with a flame ionization detector (Shimadzu, model GC-4CPF) using glass column (3 mm ID×2 m) packed with 20% diethylene glycol succinate on 60/80 mesh chromosolb WAW (Shinwa Chemical Ind.); column temperature, 190℃; and He flow rate, 40 mL/min. Each peak of fatty acid methyl esters was identified with authentic standards or analyzed

with a GC-mass spectrometer (Hewlett Packard, model HP-5900) using 1.25 m glass column packed with 2 % OV-101 on 60/80 mesh uniport HP (Gaskuro-kogyo); column temperature, 150℃; He flow rate, 30 mL/min; and ionization voltage, 70 eV (EI mode). γ-Linolenic acid methylester obtained from the lipid of *Mortierella* fungus was also identified with ^1H NMR (Hitachi, model R-40, 90 Mz) and ^{13}C NMR (Varian, model FT-80, 25.2 Mz), and also α-linolenic acid methylester obteined from linseed oil and linoleic acid methylester (Funacoshi Chemicals) were used in these analysis for comparison.

Results and Discussion

Cell growth and Lipid content. The cell growth and lipid content of 6 species (11 strains) of the order Mucorales in the class Zygomycetes are shown in **Table 1**. Each species showed a variety of cell growth and lipid content. Especially two strains of *Mortierella isabellina* were observed to have more than 50% TL content in the cells. Cultivations at 20℃ gave higher TL contents than those at 30℃, although the cell growths at 20℃ were less than a half of those at 30℃ in most strains. The TL amount of *M. isabellina* IFO 7884 cultivated at 20 ℃ was 86.1% of the dry cell weight. However, the highest production of lipid, 1.9 g/L was obtained at 30℃ with the same strain.

Neutral lipid content was almost in proportion to TL content, and in case of high TL content, more than 90% of TL was occupied with NL. Compared with NL content, PL content was varied. Polar lipid content were in a range from 3 to 6% of dry cell weight, comparatively constant values.

Table 1. Lipids in fungi of order Mucorales in Zygomycetes

Exp. No.	Organism	IFO No.	Culture Days	Temp. (℃)	DC (g/L)	TL (mg/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
1	*Abisidia corymbifere*	4410	17	30	1.41	154	10.9	9.6	1.3
2	*Abisidia corymbifere*	8084	14	30	3.18	843	26.5	22.7	3.8
3	*Choanephora circinans*	5991	15	30	5.53	337	6.1	5.2	0.9
4	*Mortierella isabellina*	7824	18	30	1.59	865	54.4	48.9	5.5
5	*Mortierella isabellina*	7824	19	20	0.66	508	76.9	68.1	8.8
6	*Mortierella isabellina*	7884	19	30	3.03	1.918	63.3	59.8	3.5
7	*Mortierella isabellina*	7884	17	20	1.31	1.128	86.1	80.2	5.9
8	*Mucor ambiguus*	6742	26	30	0.72	71	9.9	7.7	2.2
9	*Mucor ambiguus*	8092	26	30	0.97	133	13.7	7.0	6.7
10	*Rizopus oryzae*	4734	13	30	1.42	216	15.2	12.1	3.1
11	*Rizopus oryzae*	5418	12	30	1.90	600	31.6	29.2	2.4
12	*Rizopus oryzae*	5418	15	20	1.16	661	57.0	50.3	6.7
13	*Syncephalastrum racemosum*	4816	21	30	2.12	242	11.4	5.8	6.6
14	*Syncephalastrum racemosum*	4828	18	30	3.15	290	9.2	6.1	3.1

DC: Dry cell weight TL: Total lipid NL: Neutral lipid PL: Polar lipid

Table 2. Neutral lipid distribution of order Mucorales

Exp. No.	IFO No.	Neutral lipid distribution(%) TG	1,2-DG	1,3-DG	MG	FFA	SE	FS	Uk
1	4410	62.5	5.3	7.5	5.4	3.5	9.5	5.3	1.3
3	8084	71.4	13.2	7.9	2.1	4.6	Tr	0.4	0.4
4	7824	75.9	7.5	5.9	0.7	7.8	0.2	1.9	0.2
6	7884	87.9	3.4	3.7	0.5	3.7	0.5	0.4	0.0
9	8092	76.6	4.1	5.1	7.8	4.3	0.8	0.5	0.9
11	5418	83.9	3.2	3.2	5.4	1.5	0.9	0.6	1.2
13	4816	74.3	5.6	8.1	7.7	2.1	Tr	0.6	1.8
14	4828	74.9	4.8	6.8	2.6	8.8	0.1	1.0	1.1

TG:Triacylglycerol DG:Diacylglycerol MG:Monoacylglycerol FFA:Free fatty acid SE:Sterol ester FS:Free sterol Uk:Unknown substance Tr:Trace

Neutral lipid and phospolipid distribution. Neutral lipid distribution of 8 strains is shown in **Table 2**. Neutral lipid fraction was consisted of triacylglycerol, 1,2-diacylglycerol, 1,3-diacylglycerol, monoacylglycerol, free fatty acid, sterol ester, free sterol, and unknown substance whose Rf was between free sterol and monoacylglycerol in TLC analysis. The most prominent NL was triacylglycerol, content of which was in a range of 62 - 88% of total NL. Triacylglycerol content was varied considerably depending on the cell growth and TL content. The strain, which accumulate large amount of lipid, showed high content of TG. The content of diacylglycerol and monoacylglycerol were in wider range by strains compared with the content of triacylglycerol.

Table 3. Phospholipid distribution of order Mucorales

Exp. No.	IFO No.	Temp. (℃)	Pospholipid distribution (%) PC	PE	PS	PI	PG	LPC	CL	p-GL
1	4410	30	26.1	10.1	0.0	1.9	0.0	57.5	0.0	0.0
2	8084	30	74.8	6.8	4.2	5.1	9.0	0.0	0.0	0.0
3	5991	30	42.5	40.6	3.5	3.3	6.6	0.0	3.4	0.0
4	7824	30	58.3	20.5	3.6	1.8	5.4	1.4	3.0	4.1
5	7824	20	62.6	21.3	2.8	2.1	4.0	1.1	5.0	1.0
6	7884	30	53.2	15.2	11.0	20.6	0.0	0.0	0.0	0.0
7	7884	20	38.5	14.9	5.5	27.6	0.0	4.6	6.4	2.3
8	6742	30	5.9	29.9	2.9	0.0	2.3	28.1	13.9	17.1
9	8092	30	37.5	49.9	3.1	2.0	3.5	1.0	3.2	3.2
10	4734	30	53.8	5.0	13.1	5.5	8.0	18.8	1.0	0.0
11	5418	30	52.1	3.2	8.9	8.8	0.0	10.1	8.1	0.0
12	5418	20	52.2	3.4	17.2	1.3	0.0	22.6	3.3	0.0
13	4816	30	18.2	49.1	8.9	8.7	0.0	3.4	11.6	0.0
14	4828	30	22.4	42.7	4.7	10.6	3.8	5.3	8.4	2.1

PC:Phosphatidylcholine PE:Phosphatidylethanolamine PS:Phosphatidylserine PI:Phosphatidylinositol PG:Phosphatidylglycerol LPC:Lysophosphatidylcholine CL:Cardiolipine p-GL:Phosphorus containing glycolipid

Phospholipid distribution of 11 strains is shown in **Table 3**. Phospholipid fraction was consisted of phosphatidylcholine (PC), phospatidyl-ethanolamine, phosphatidylserine, phosphatidylinositol, lysophosphatidyl-choline (LPC), phospatidylglycerol, and cardiolipin. The polar lipid also included phosphorus glycolipid (p-GL) as unknown structure. In these strains, PC was principal ingredients, however, phospholipid distribution were divergent with respect to strains and growth temperature. In the strains of IFO 4410 and IFO 6742, high content of LPC as well as low content of PC were shown. In the present study, it is not clarified that, the diversity of NL distribution and phospholipid distribution depended upon strains or cultural conditions.

Fatty acid composition. The fatty acid composition of NL and PL of 11 strains is shown in **Table 4**. The linolenic acid (18:3) included in these strains was identified as GLA by the GC-mass spectra and NMR analysis as shown by Shaw[20]. Namely, linolenic acid methyl of *M. isabellina* IFO 7824 and α-linolenic acid methyl showed the same molecular ion peak of m/e 292 with GC-mass analysis, though they showed different retention time in GC analysis. And also spectra with ^{1}H NMR and ^{13}C NMR proved that the linolenic acid obtained from these strains was the octadecatrienoic acid which had *cis*-6, *cis*-9, *cis*-12 double bonds.

Table 4. Fatty acid compositions of lipids in fungi of order Mucorales

Exp. No.	IFO No.	Temp. (℃)	Lipid	Fatty acid composition (%) 14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3*	20:0	20:1	20:2	20:4
1	4010	30	NL	0.7	0.2	24.0	0.5	0.1	0.2	6.7	45.7	7.8	10.4	0.5	0.1	0.1	0.0
			PL	1.8	0.1	21.5	0.6	0.1	0.0	3.0	42.4	10.7	16.3	0.4	0.4	0.4	0.0
2	8084	30	NL	1.0	0.4	12.8	0.7	0.4	0.0	19.3	26.0	18.8	2.7	0.2	0.3	0.0	0.0
			PL	5.0	0.2	15.7	1.6	0.0	0.3	7.6	20.5	34.7	3.2	0.3	0.0	0.0	0.1
3	5991	30	NL	1.1	0.5	25.0	2.0	0.0	0.0	14.2	33.9	16.5	2.8	0.3	0.0	0.1	0.1
			PL	0.7	0.3	15.4	2.4	0.0	0.4	4.8	38.4	29.5	5.9	0.2	0.0	0.2	0.0
4	7824	30	NL	0.6	0.1	21.4	0.0	1.4	0.1	4.1	49.0	10.3	10.7	0.1	0.0	Tr	0.0
			PL	0.5	0.0	13.2	0.0	1.6	0.2	1.3	40.9	18.6	21.5	0.0	0.0	0.6	0.0
5	7824	20	NL	0.9	0.3	26.3	2.0	0.0	0.3	4.1	45.4	10.6	8.2	0.0	0.1	Tr	0.0
			PL	1.2	0.5	17.5	3.6	0.0	1.2	2.1	42.0	13.8	10.5	0.0	0.2	0.0	0.0
6	7884	30	NL	1.5	0.1	35.9	5.8	0.0	0.0	2.8	41.7	7.3	4.2	Tr	0.0	0.0	0.0
			PL	0.8	0.1	10.9	6.6	0.0	0.4	1.0	39.5	21.3	13.0	0.0	0.0	0.0	0.0
7	7884	20	NL	1.5	0.1	33.5	5.7	0.0	0.1	2.0	43.9	8.8	3.5	0.1	Tr	0.0	0.0
			PL	1.2	0.1	26.3	6.1	0.0	0.3	1.7	44.2	13.2	5.1	0.2	Tr	0.0	0.0
8	6742	30	NL	7.9	0.5	13.2	9.2	0.0	0.2	15.5	23.8	12.1	7.7	0.2	0.0	0.1	0.0
			PL	2.0	0.2	13.0	9.3	0.0	0.3	7.7	36.6	15.5	8.7	0.3	0.0	0.8	0.0
9	8092	30	NL	8.8	0.5	17.0	5.3	0.0	0.2	13.1	26.5	9.1	4.9	0.6	3.6	0.4	0.2
			PL	2.9	0.4	11.6	8.2	0.0	0.4	6.2	37.0	15.3	10.4	0.0	0.1	0.3	0.0
10	4734	30	NL	0.7	0.7	20.3	1.4	0.1	0.2	11.4	36.9	17.1	4.9	0.2	0.1	0.5	0.0
			PL	1.0	0.6	15.0	2.1	0.0	0.4	4.1	37.2	18.7	5.4	0.3	0.0	0.0	0.0
11	5418	30	NL	0.5	0.2	20.7	1.9	0.0	0.1	8.0	48.9	10.8	6.3	0.1	0.0	0.0	0.0
			PL	1.6	0.3	16.2	1.5	0.0	0.6	3.5	33.3	21.1	15.3	0.3	0.0	0.4	0.6
12	5418	20	NL	0.5	0.1	22.2	1.7	0.0	0.0	7.1	51.3	7.8	5.6	Tr	0.0	0.1	0.0
			PL	0.6	0.3	15.9	1.2	0.0	0.4	2.0	38.9	21.6	13.1	0.0	0.0	0.2	0.0
13	4816	30	NL	1.2	0.0	17.4	1.0	0.0	0.0	7.5	41.6	14.3	12.5	0.0	0.2	0.0	0.0
			PL	0.7	0.0	20.3	1.2	0.0	0.0	4.5	49.7	11.3	10.8	0.0	0.3	0.2	0.0
14	4828	30	NL	0.5	0.1	18.1	0.6	0.0	0.6	17.0	40.9	9.0	9.1	0.0	0.2	0.1	0.1
			PL	0.5	Tr	12.8	0.6	0.0	0.1	6.5	46.4	10.5	15.7	0.0	0.1	0.6	0.0

NL:Neutral lipid PL:Polar lipid UFA:Total unsaturated fatty acids Tr:Trace *γ-Linolenic acid

γ-Linolenic acid content was higher in PL than in NL with almost all strains. The strains of *A. corymbifera* IFO 4010, *M. isabellina* IFO 7824, *S. racemosum* IFO 4816 showed more than 10% GLA content in both NL and PL fractions. The two strains of *Mucor ambiguus* showed higher levels of

myristic acid and palmitoleic acid than the other strains. Polyunsaturated fatty acids with 20 carbon atoms were not detected in these strains.

It is well known that GLA is an intermediate in the transformation of linoleic acid into arachidonic acid, principle precursor of prostaglandins, which play very important roles in the human body. On the other hand, GLA dose not occur in general plant oils. Therefore, *Mortierella isabellina* which has high lipid productivity and relatively high content of GLA is expected to be excellent source for production of GLA.

Summary

The lipid productivities of 6 species (11 strains) of the order Mucorales in the class Zygomycetes were investigated. Furthermore, the distribution of lipid and fatty acid composition were determined. The maximum lipid content, 86.1%, was obtained with *Mortierella isabellina* IFO 7884 at 20℃. However, the highest lipid production of 1.9 g/L, was obtained at 30℃ with the same strain. Linolenic acid included in the strain was estimated as *cis*-6, *cis*-9, *cis*-12 octadecatrienoic acid (γ-linolenic acid, GLA) with the GC-mass and NMR spectra. From the point of GLA content, the strains of *A. corymbifera* IFO 4010, *M. isabellina* IFO 7824, and *S. racemosum* IFO 4816 produced showed more than 10% GLA content in both NL and PL fractions. *Mortierella isabellina* which has high lipid productivity and relatively high content of GLA is expected to be excellent source for production of GLA.

Section 2 Lipid composition of genus *Mortierella* grown on glucose and *n*-decane as carbon source

Some microorganisms can utilize *n*-paraffin as carbon source as well as glucose. There are many reviews or studies on usability of *n*-paraffin as carbon source for microorganisms by Iizuka or Fukui *et.al.*[39,40]. There are many microorganisms which can use liquid *n*-paraffins with carbon chain length from C5 to C19. The study on usability of *n*-paraffins as carbon source for microorganisms was started in 1960's to produce microbial proteins, and later to produce amino acid, organic acid, fatty acid, and intermediate of vitamins[41-44]. Misaim *ethereal.*[45,46] examined production of lipid using *n*-paraffins by yeast of *Candid*, and they described the changes of lipid and fatty acid compositions. Iizuka *et.al.*[47], applied a patent on arachidonic acid production by a *Mucor* fungus.

The author searched fungi which have high lipid productivity for the purpose of production of lipid by microorganism, and found that the two strains of *Mortierella isabellina*, IFO 7884 and IFO 7824 have high lipid productivity and relatively high content of GLA grown on glucose as carbon source as described in the previous section.

This section deals with the investigation of the cell growth, lipid production, fatty acid composition and lipid distribution of 33 strains of the genus *Mortierella* which was not investigated so far, using glucose or *n*-decane as carbon source at a purpose of search for a strain suitable for lipid or GLA production.

Materials and Methods

Microorganisms and cultural conditions. *Mortierella* genus fungi, 33 strains, were obtained from the Culture Collection of the Institute of

Fermentation, Osaka. The liquid culture medium using glucose as carbon source contained the followings; 30 g glucose, 3.0 g KH_2PO_4, 3.0 g NH_4NO_3, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnSO_4 \cdot 4H_2O$, 2.0 mg Thiamin-HCl and 0.02 mg D-Biotin in 1000 mL of distilled water. *n*-Decane medium contained the followings; 9.1 g *n*-decane, 2.0 g KH_2PO_4, 0.91 g NH_4NO_3, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 2.0 mg Thiamin-HCl, 0.02 mg D-Biotin and 0.2 mL Tween-20 in 1000 mL of distilled water. Initial pH of each medium were adjusted to 4.6. The microorganisms were grown in 1000 mL erlenmyer flasks containing 400 mL liquid medium at 20℃ or 30℃ under rotary shaking (150 rpm) for 10 days.

Analytical methods. Analytical methods were performed as described previously in the section 1 of this chapter. The cell growth was determined by dry cell weight. Lipid was extracted from the mycelium, and the total lipid production was measured by weight. The total lipid was fractionated into neutral lipid (NL) and polar lipid (PL) by silicic acid chromatography. Fatty acid composition of each lipid fraction was analyzed with gas chromatograph equipped with a flame ionization detector. Neutral lipid distribution was analyzed by TLC as described in the previous section. Polar lipid distribution was analyzed by high-performance liquid chromatography (HPLC) (Simadzu, model LC-3A) using UV detector (absorbance 203 nm) with internal standard as described by the authors[48]. The chromatography was performed on a system combined with two columns (Zorbax CN and Zorbax ODS) using acetonitrile/water/phosphoric acid (100:2:0.1 by vol.) as eluent.

Table 1. Cell growth and lipid formations from glucose in 33 strains of genus *Mortierella*

Exp. No.	Organism	IFO No.	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
1	*M. alpina*	8568	30	Tr	-	-	-	-
2	*M. bainieri*	8569	30	Tr	-	-	-	-
3	*M. elongata*	8570	30	0.15	-	-	-	-
4	*M. exigua*	8571	30	Tr	-	-	-	-
5	*M. humicola*	8188	30	1.15	-	-	-	-
6	*M. humicola*	8288	30	1.35	-	-	-	-
7	*M. humicola*	8289	30	0.75	-	-	-	-
8	*M. hygrophila*	5941	30	Tr	-	-	-	-
9	*M. isabellina*	6739	30	3.33	1.12	33.6	31.3	2.3
10	*M. isabellina*	7873	30	5.45	2.19	40.2	37.2	3.0
11	*M. isabellina*	7873	20	6.18	1.55	25.1	21.8	3.3
12	*M. isabellina*	7874	30	4.13	1.49	36.1	33.1	3.0
13	*M. isabellina*	8183	30	9.08	3.38	37.2	36.1	1.1
14	*M. isabellina*	8183	20	9.63	3.55	36.9	34.7	2.2
15	*M. isabellina*	8572	30	2.54	1.25	49.1	47.5	1.6
16	*M. isabellina*	8286	30	2.73	0.93	34.0	32.0	2.0
17	*M. isabellina*	8309	30	5.25	1.30	24.7	22.9	1.8
18	*M. isabellina*	8308	30	6.28	2.55	40.7	38.8	1.9
19	*M. isabellina*	8308	20	10.10	5.02	49.7	48.1	1.6
20	*M. vinacea*	6738	30	5.03	1.85	36.8	33.7	3.1
21	*M. vinacea*	6738	20	3.03	0.61	20.0	17.1	2.9
22	*M. vinacea*	7875	30	1.58	0.21	13.6	11.8	1.8
23	*M. minutissima*	8573	30	Tr	-	-	-	-
24	*M. nana*	8190	30	0.78	-	-	-	-
25	*M. nana*	8794	30	4.53	2.32	51.3	49.2	2.1
26	*M. nana*	8794	20	3.30	0.90	27.3	25.4	1.9
27	*M. nana*	8795	30	0.20	-	-	-	-
28	*M. parvispora*	8574	30	Tr	-	-	-	-
29	*M. polycepala*	6335	30	Tr	-	-	-	-
30	*M. ramanniana*	7825	30	1.05	-	-	-	-
31	*M. ramanniana*	8184	30	2.68	0.97	36.2	34.5	1.7
32	*M. ramanniana*	8185	30	2.45	0.87	35.6	33.7	1.9
33	*M. ramanniana*	8287	30	3.65	1.49	40.9	39.4	1.5
34	*M. ramanniana*	8287	20	1.98	0.71	35.8	33.4	2.4
35	*M. ramanniana var. angulispora*	5426	30	2.80	0.38	13.6	10.7	2.9
36	*M. ramanniana var. angulispora*	6744	30	3.20	0.43	13.4	11.0	2.4
37	*M. ramanniana var. angulispora*	8186	30	2.43	0.84	34.8	31.7	3.1
38	*M. ramanniana var. angulispora*	8186	20	6.33	2.23	35.2	34.0	1.2
39	*M. ramanniana var. angulispora*	8187	30	3.80	0.71	18.8	17.4	1.4
40	*M. ramanniana var. angulispora*	8187	20	7.45	1.82	24.4	21.8	2.6
41	*M. verticillata*	8575	30	Tr	-	-	-	-

DC:Dry cell weight TL:Total lipid NL:Neutral lipid PL:Polar lipid Tr:Trace

Results and Discussion

Cell growth and lipid production of genus Mortierella using glucose and n-decane. Cell growth and lipid formations of 33 strains of genus *Mortierella* grown on glucose as carbon source is shown in **Table 1.** Many strains of *M. isabellina* showed high cell growth on the glucose, and there lipid content were as high as 40%. Two strains of IFO 8183 and IFO 8308 reached particularly high lipid production of more than 3 g/L total lipid. The maximum cell growth of 10.10 g/L and the maximum lipid production of 5.03 g/L were obtained with the strain of IFO 8308 at 20℃, whose value were larger than that value obtained from the strain of IFO 7884 as well as previously investigated. The other strains which showed relatively high cell growth were *M. vinacea* IFO 6738, *M. nana* IFO 8794, and two strains of *M. ramanniana* var. *angulispora* IFO 8186 and IFO 8187. The cultural temperature which gave high lipid accumulation was varied among a strain. Total lipid content differed from 10% to 50% by a strain. Contents of PL ranged from 1.2% to 3.3%, relatively constant.

Table 2. Cell growth and lipid formations from *n*-decane in 12 strains of genus *Mortierella*

Exp. No.	Organism	IFO No.	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
42	*M. humicola*	8288	30	0.23	-	-	-	-
43	*M. humicola*	8289	30	0.48	-	-	-	-
44	*M. isablellina*	6739	30	2.38	0.75	31.5	26.1	5.4
45	*M. isablellina*	6739	20	1.75	0.35	19.9	11.6	8.3
46	*M. isablellina*	7873	30	1.63	0.28	17.3	10.3	7.0
47	*M. isablellina*	7873	20	0.50	-	-	-	-
48	*M. isablellina*	7874	30	1.45	0.25	17.5	10.8	6.7
49	*M. isablellina*	7874	20	1.45	0.22	15.1	8.7	6.4
50	*M. isablellina*	8183	30	2.38	0.47	19.8	12.5	7.3
51	*M. isablellina*	8183	20	1.80	0.34	18.9	12.7	6.2
52	*M. vinacea*	6738	30	0.85	0.11	13.5	9.4	4.1
53	*M. vinacea*	6738	20	0.60	0.05	8.9	4.9	4.0
54	*M. nana*	8190	30	0.18	-	-	-	-
55	*M. nana*	8794	30	1.53	0.31	20.6	12.7	7.9
56	*M. nana*	8794	20	0.65	-	-	-	-
57	*M. ramanniana*	7825	30	0.50	-	-	-	-
58	*M. ramanniana*	8184	30	0.48	-	-	-	-
59	*M. ramanniana Var. angulispora*	8187	30	0.50	0.13	26.3	17.1	9.2
60	*M. ramanniana Var. angulispora*	8187	20	0.90	0.21	23.4	11.3	12.1

DC:Dry cell weight TL:Total lipid NL:Neutral lipid PL:Polar lipid

Cell growth and lipid formations on *n*-decane of 12 strains which grew well on glucose is shown in **Table 2**. On *n*-decane culture, 4 strains of *M.*

isabellina also grew well similar to the glucose culture. *M. isabellina* IFO 6739 gave the highest cell growth and lipid production. Growth on *n*-decane showed higher content of PL, ranged from 4.0 to 12.1%, than those on glucose by the same strains.

Table 3. Fatty acid composition of neutral lipid and polar lipid of genus *Mortierella* grown on glucose as carbon source

Exp. No.	IFO No.	Temp. (℃)	Lipid	Fatty acid composition (%) 14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3 *	UFA (%)
9	6739	30	NL	0.9	0.3	22.0	3.0	-	0.3	5.1	55.8	7.8	3.8	70.7
			PL	1.5	0.2	25.1	3.5	0.6	0.5	4.0	51.6	9.2	3.4	68.2
10	7873	30	NL	1.0	0.1	26.0	1.6	-	-	6.9	49.4	10.3	4.7	66.0
			PL	0.5	0.1	11.1	1.7	0.1	-	1.6	43.4	27.0	14.0	86.1
11	7873	20	NL	1.7	0.2	28.3	2.3	-	0.1	6.1	41.6	10.5	9.0	63.5
			PL	0.5	0.1	11.7	1.9	-	0.1	1.5	38.2	23.8	22.3	86.3
12	7874	30	NL	1.5	0.3	33.3	1.0	-	-	5.4	46.0	9.0	3.5	59.5
			PL	1.7	0.4	25.4	2.3	1.2	0.6	3.7	44.4	15.1	4.2	66.6
13	8183	30	NL	1.1	-	27.6	2.0	-	-	5.0	52.4	8.0	3.9	66.3
			PL	0.3	-	9.6	2.2	-	-	0.7	43.9	26.9	16.5	89.5
14	8183	20	NL	0.9	Tr	24.2	1.8	-	-	5.4	55.0	7.9	4.5	69.2
			PL	0.5	0.1	11.7	1.9	-	0.1	1.5	38.2	23.8	22.3	86.3
17	8309	30	NL	0.4	0.1	15.9	1.9	-	-	4.0	58.4	8.1	9.4	77.8
			PL	0.2	0.1	8.4	1.3	-	-	1.0	55.8	17.4	14.5	89.0
18	8308	30	NL	0.8	0.1	30.3	2.4	0.4	-	5.1	53.2	4.3	3.2	63.1
			PL	1.4	0.2	27.5	2.4	1.6	0.8	4.0	46.1	9.4	3.8	62.5
19	8308	20	NL	1.0	0.1	33.4	4.4	-	0.2	3.6	47.5	6.3	3.4	61.8
			PL	0.2	-	11.8	3.7	-	0.2	0.5	34.5	33.5	15.6	87.5
20	6738	30	NL	0.7	0.2	27.9	0.3	0.2	-	12.7	48.0	5.9	3.6	57.8
			PL	1.4	0.3	28.3	0.4	0.6	0.5	8.8	46.7	7.7	3.3	58.6
21	6738	20	NL	0.7	0.1	27.8	0.4	-	-	12.1	42.6	10.1	5.4	58.5
			PL	0.4	0.1	13.9	1.3	-	0.1	3.1	25.2	36.8	19.1	82.5
25	8794	30	NL	0.6	-	25.1	0.1	-	-	16.6	44.4	8.5	4.6	57.6
			PL	0.4	-	11.0	0.7	-	-	2.8	40.4	30.7	13.8	85.6
26	8794	20	NL	0.7	0.1	27.2	0.2	-	Tr	11.2	43.2	11.6	5.4	60.4
			PL	0.4	-	18.5	1.1	-	-	3.2	36.0	28.8	12.1	78.0
33	8287	30	NL	1.6	0.1	29.7	1.9	-	-	5.4	47.0	8.5	5.4	62.8
			PL	1.1	0.2	15.4	1.7	0.4	-	1.9	48.1	19.0	11.8	80.6
34	8287	20	NL	1.3	0.1	30.8	1.7	-	-	5.3	45.7	8.8	6.3	62.5
			PL	0.6	-	14.4	0.9	-	-	1.3	19.7	34.7	28.4	83.7
37	8186	30	NL	0.8	0.1	20.9	1.3	0.4	-	7.8	53.9	8.3	5.2	68.7
			PL	1.8	0.2	26.6	1.6	1.5	0.5	7.4	44.1	10.9	4.8	61.9
38	8186	20	NL	0.8	-	21.8	1.4	-	-	4.3	56.7	9.1	5.8	73.0
			PL	0.4	-	14.1	1.2	-	0.1	1.9	19.6	37.7	24.4	83.0
39	8187	30	NL	0.8	-	30.0	0.2	-	-	9.0	42.7	9.6	7.7	60.2
			PL	0.4	-	12.3	0.8	-	-	1.8	33.0	27.3	23.6	84.7
40	8187	20	NL	0.8	0.1	32.0	0.4	-	-	7.8	44.4	8.2	6.5	59.5
			PL	0.2	-	12.1	1.1	0.1	-	1.6	40.6	24.5	19.7	85.9

* γ-Linolenic acid UFA:Total unsaturated fatty acid NL:Neutral lipid
PL:Polar lipid Tr:Trace

Fatty acid compositions of neutral and polar lipid of genus Mortierella.

Fatty acid compositions of NL and PL grown on glucose as carbon sources

are shown in **Table 3**. The main fatty acids occurred were palmitic acid, stearic acid, oleic acid, linoleic acid and GLA. Polar lipid had higher degree of unsaturation and especially high content of GLA. The content of GLA differed from 3.2% to 9.4% of NL and from 3.4 to 28.4% of PL. Among the strains of *M. isabellina*, content of GLA in NL differed from 3.2% with IFO 8308 to 9.4% with IFO 8309. Three strains of *M. ramanniana* and *M. ramanniana* var. *anglispora* showed relatively high content of GLA, above 5% in NL.

Table 4. Fatty acid composition of neutral lipid and polar lipid of genus *Mortierella* grown on *n*-decane as carbon source

Exp. No.	IFO No.	Temp. (℃)	Lipid	Fatty acid composition (%) 14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3 *	UFA (%)
44	6739	30	NL	3.1	0.8	42.2	3.0	0.8	1.0	6.4	20.6	7.4	14.5	46.5
			PL	2.1	0.5	18.3	4.2	-	0.7	1.3	34.4	19.2	17.5	76.0
45	6739	20	NL	2.3	1.1	34.6	3.1	0.6	0.4	4.7	24.0	9.9	19.3	56.7
			PL	1.2	0.5	17.0	3.0	-	0.6	1.4	22.3	19.8	33.4	79.1
46	7873	30	NL	4.8	1.3	38.4	2.1	0.4	0.2	6.9	18.8	8.2	19.0	48.3
			PL	2.4	1.7	24.6	2.1	0.4	0.8	2.6	19.8	17.1	28.5	68.3
48	7874	30	NL	1.5	0.9	30.2	3.8	0.6	0.4	5.3	34.5	11.5	11.2	61.4
			PL	1.0	0.9	17.0	3.3	-	0.3	1.3	36.4	20.2	19.2	79.4
49	7874	20	NL	2.0	1.0	32.8	4.8	-	1.1	6.4	22.0	8.9	20.9	57.7
			PL	1.2	1.0	25.7	4.1	-	0.6	2.7	20.4	13.8	30.4	69.3
50	8183	30	NL	1.8	1.5	32.8	4.5	-	1.1	4.3	23.1	7.1	23.7	59.5
			PL	1.5	1.3	22.5	4.7	-	0.6	1.2	30.1	10.6	27.1	73.1
51	8183	20	NL	2.1	0.7	29.8	5.0	-	-	3.8	26.7	6.4	24.9	63.0
			PL	1.5	1.4	18.8	3.9	-	0.7	1.5	22.6	10.9	38.3	76.4
52	6738	30	NL	2.2	0.8	32.7	3.5	0.6	0.7	3.9	27.8	9.9	17.7	59.6
			PL	3.7	0.9	29.0	3.1	0.6	0.9	2.5	22.1	11.5	25.6	63.2
53	6738	20	NL	2.6	1.2	45.2	0.5	-	-	4.6	18.6	6.9	19.2	45.2
			PL	2.6	0.9	24.5	2.3	0.9	1.2	2.5	11.4	13.0	40.3	68.2
55	8794	30	NL	1.6	1.6	32.8	4.0	3.6	3.0	4.2	19.0	9.1	20.3	55.4
			PL	1.7	1.2	18.5	3.8	-	1.4	2.4	20.8	17.3	32.8	76.1
59	8187	30	NL	3.0	1.5	39.3	0.4	0.7	0.2	7.4	19.2	8.2	20.2	48.2
			PL	4.7	2.0	25.8	2.7	1.1	1.0	2.4	18.3	10.5	30.6	63.1
60	8187	20	NL	3.4	1.4	32.3	1.8	0.6	0.1	6.1	20.4	5.8	28.3	56.4
			PL	2.9	1.3	24.6	1.5	0.4	0.4	2.1	21.2	9.0	35.9	68.0

* γ-Linolenic acid UFA:Total unsaturated fatty acid NL:Neutral lipid
PL:Polar lipid Tr:Trace

Fatty acid compositions of NL and PL grown on *n*-decane as carbon sources are shown in **Table 4**. *n*-Decane culture showed higher content of GLA (14.5% to 28.2%) and palmitic acid in NL than those with glucose culture. Fatty acid composition of PL showed high level of GLA (17.5% to

40.3%) but lower level of oleic acid, resulting in the highest content of GLA in many strains. Contents of linoleic acid, on the other hand, were lower than those in glucose culture, indicating that *n*-decane culture has some acceleration of Δ6 desaturase activity which converts linoleic acid to GLA compared with glucose culture. *M. ramanniana* var. *angulispora* which showed high content of GLA in glucose culture also showed GLA content in *n*-decane culture. The strains which showed low content of GLA on glucose , also showed low GLA content on *n*-decane. Content of GLA seems to be dependent of the strain more than carbon source.

Neutral and polar lipid distributions of genus Mortierella. Neutral lipid distribution of glucose and *n*-decane culture are shown in **Table 5** and **Table 6**, respectively. Triacylglycerol content in glucose culture and *n*-decane culture ranged from 80.0% to 96.7% and from 48.1% to 84.1%, respectively. Culture on *n*-decane was characteristic of higher contents of diacylglycerol and free sterol. It showed that the lipid accumulated as triacylglycerol was lower in *n*-decane culture.

Table 5. Neutral lipid distribution of genus *Mortierella* grown on glucose as carbon source

Exp. No.	IFO No.	Temp. (℃)	Neutral lipid (%) TG	1,2-DG	1,3-DG	MG	FFA	SE	FS
9	6739	30	84.5	8.2	5.4	0.3	0.9	0.2	0.6
10	7873	30	80.0	5.7	3.2	2.1	5.6	1.5	1.2
11	7873	20	81.9	9.2	2.8	1.5	0.8	2.4	1.2
13	8183	30	92.0	3.2	0.5	1.0	1.3	1.3	0.7
14	8183	20	94.2	2.6	0.6	0.3	0.3	1.5	0.7
18	8308	30	93.8	3.0	2.5	Tr	0.4	0.2	0.2
19	8308	20	95.4	1.6	1.3	0.2	0.3	0.6	0.5
20	6738	30	86.2	7.7	4.3	Tr	1.0	0.2	0.6
21	6738	20	86.9	8.1	1.6	0.2	0.7	1.0	1.5
25	8794	30	91.8	3.6	1.5	0.3	1.5	0.2	1.1
26	8794	20	89.2	4.2	5.0	0.3	0.2	0.7	0.5
34	8287	20	86.7	9.7	1.5	0.1	0.2	0.8	1.0
38	8186	20	96.7	1.1	0.8	0.2	0.3	0.5	0.4
39	8187	30	80.8	5.0	5.8	1.1	4.6	1.5	1.2
40	8187	20	90.1	3.1	2.8	0.7	2.0	0.9	0.4

TG:Triacylglycerol DG:Diacylglycerol MG:Monoacylglycerol
FFA:Free fatty acid SE:Sterol ester FS:Free sterol Tr:Trace

Table 6. Neutral lipid distribution of genus *Mortierella* grown on *n*-decane as carbon source

Exp. No.	IFO No.	Temp. (℃)	Neutral lipid (%) TG	1,2-DG	1,3-DG	MG	FFA	SE	FS
44	6739	30	80.1	12.8	3.5	-	1.2	0.3	2.0
45	6739	20	54.2	30.6	5.6	0.4	1.1	0.9	6.8
48	7874	30	79.0	12.4	2.5	1.8	0.2	0.6	3.4
49	7874	20	67.6	22.9	1.4	3.0	1.2	0.3	3.5
50	8183	30	48.1	30.7	4.9	3.1	1.1	0.9	10.7
51	8183	20	72.4	15.2	2.5	2.0	2.1	0.6	5.1
52	6738	30	73.4	15.0	3.9	-	1.5	0.4	5.6
53	6738	20	60.8	21.7	5.3	0.4	3.9	0.4	7.2
55	8794	30	84.1	6.3	2.2	Tr	1.1	0.7	5.2
59	8187	30	63.7	21.7	5.4	-	3.6	0.6	4.6

TG:Triacylglycerol DG:Diacylglycerol MG:Monoacylglycerol
FFA:Free fatty acid SE:Sterol ester FS:Free sterol Tr:Trace

Polar lipid distribution of glucose and *n*-decane culture are shown in **Table 7** and **Table 8**, respectively. In each strain on both glucose and *n*-decane, dominant components were phosphatidylcoline and phosphatidylethanolamine. These two components reached 60% in PL.

Table 7. Polar lipid distribution of genus *Mortierella* grown on glucose as carbon source

Exp. No.	IFO No.	Temp. (℃)	Polar lipid (%) PC	PE	PS	PI	LPC	LPE	p-GL	GL	TPL (%)
10	7873	30	45.0	21.6	1.1	6.0	-	-	4.6	1.0	79.3
11	7873	20	35.0	25.3	2.3	4.4	-	0.2	3.8	5.5	76.5
13	8183	30	37.2	17.2	2.3	6.1	-	-	4.9	11.3	79.0
14	8183	20	45.7	23.5	4.9	1.8	-	-	3.2	4.3	83.4
21	6738	20	36.0	24.6	0.8	2.0	-	1.4	3.7	3.2	71.7
25	8794	30	50.6	19.0	1.5	4.6	-	-	2.6	8.9	87.2
34	8287	20	25.0	37.1	1.5	3.3	1.0	-	5.0	5.8	78.7
38	8186	20	34.3	21.0	2.7	4.0	-	-	3.6	6.3	71.9
39	8187	30	31.3	29.3	1.5	8.8	-	-	5.8	12.8	89.5
40	8187	20	27.9	21.9	0.8	3.7	10.1	-	3.0	7.8	75.2

PC:Phosphatidylcholine PE:Phosphatidylethanolamine PS:Phosphatidylserine PI
Phosphatidylinositol LPC:Lysophosphatidylcholine LPE:Lysophosphatidylethanola
p-GL:Phosphorus containing glycolipid GL:Glycolipid TPL:Total polar lipids c
by HPLC using internal standerd

Table 8. Polar lipid distribution of genus *Mortierella* grown on *n*-decane as carbon source

Exp. No.	IFO No.	Temp. (℃)	Polar lipid (%) PC	PE	PS	PI	LPC	LPE	p-GL	GL	TPL (%)
44	6739	30	34.3	16.2	1.4	9.0	-	-	4.9	9.2	75.0
45	6739	20	45.8	18.0	3.2	9.9	-	-	4.1	3.6	84.6
48	7874	30	41.4	22.0	2.4	6.9	-	-	3.7	8.0	84.4
49	7874	20	42.5	23.4	0.7	7.5	-	0.8	3.7	4.1	82.7
50	8183	30	25.3	15.5	1.3	5.2	-	-	3.0	5.6	55.9
51	8183	20	40.4	22.0	2.9	7.0	-	-	2.4	4.3	79.0
52	6738	30	40.7	16.3	2.9	6.0	-	0.2	2.9	8.6	77.6
53	6738	20	48.5	21.0	0.5	4.0	-	0.2	2.6	6.8	83.6
55	8794	30	36.8	24.8	1.3	4.0	-	0.2	2.3	5.5	74.9
59	8187	30	44.9	25.1	0.8	9.5	-	-	4.3	5.1	89.7

PC:Phosphatidylcholine PE:Phosphatidylethanolamine PS:Phosphatidylserine PI Phosphatidylinositol LPC:Lysophosphatidylcholine LPE:Lysophosphatidylethanola p-GL:Phosphorus containing glycolipid GL:Glycolipid TPL:Total polar lipids c by HPLC using internal standerd

Summary

Determination of the lipid production and lipid distribution of 33 strains of genus *Mortierella* grown on glucose and *n*-decane as carbon sources were carried out. The maximum accumulation of lipid, 5.03 g/L-medium and 49.7% of lipid content were obtained in the strain of *M. isabellina* IFO 8308 grown on glucose as carbon source at 20℃. Fatty acid compositions and lipid distributions of NL and PL were determined. Neutral lipid on *n*-decane culture showed higher content of GLA (14.5% to 28.2%) compared with those in glucose culture. Fatty acid composition of PL showed high content of GLA (17.5% to 40.3%) but lower content of oleic acid, consequently, GLA became the fatty acid of the highest content in many strains. The content of GLA in the strain of *M. ramanniana* var. *angulispora* IFO 8187 was high value under each cultural condition using either glucose or *n*-decane as carbon source. The strains which showed low content of GLA on glucose , also showed low content it on *n*-decane. Content of GLA seems to be dependent of the strain more than carbon source.

CHAPTER II

Influence of Cultural Conditions on Lipid Composition and Lipid Productivity

Section 1 Influence of cultural conditions on lipid compositions of the two strains of *Mortierella isabellina*

The cultivation factors, especially carbon source, nitrogen source, and culture temperature affect the cell growth, lipid accumulation, lipid distribution, and fatty acid composition. However, the research of lipid production using Zygomycetes has been few compared with the research of lipid production using oleaginous yeast[19,30,31]. Zygomycetes fungi contains some polyunsaturated fatty acids like γ-linolenic acid (GLA), dihomo-γ-linolenic acid and arachidonic acid[20,21], and was expected to production of polyunsaturated fatty acids owing to its characteristic fatty acid composition . The author searched for a fungus which have high lipid productivity for the purpose of lipid production by microorganisms. On the screening of fungi, *Mortierella isabellina* was found to accumulate a large amount of lipid and relatively high content of GLA among the Zygomycetes fungi was described previously[Chapter I, section 1].

This section deals with the investigation of the influence of cultural conditions, especially the ratio of carbon to nitrogen atom weight (C/N ratio) in a medium, nitrogen source, and cultivation temperature on cell growth and lipid accumulation in the mycelium of *M. isabellina*.

Materials and Methods

Microorganisms and cultural conditions. Two strains of *Mortierella isabellina* IFO 7884 and 7824 were used in this study. Basically, the

liquid culture medium contained the followings; 30 g glucose, 3.0 g KH_2PO_4, 3.0 g NH_4NO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 2.0 mg Thiamin-HCl and 0.02 mg D-Biotin in 1000 mL of distilled water. Initial pH of each medium was adjusted to 4.6. The C/N ratio of the medium was 11.4. Mediums of the different C/N ratio at 5.7, 114 and 343 were prepared by the addition of 6.0 g, 0.3 g and 0.1 g NH_4NO_3, respectively. Media with different nitrogen source was prepared by adding 2.3 g of urea, 3.1 g of ammonium sulfate or 4.0 g of potassium nitrate at 11.4 of C/N ratio. The microorganisms were grown in 1000 mL erlenmyer flasks containing 400 mL liquid medium at various temperatures under rotary shaking (150 rpm).

Analytical methods. Analytical methods were performed as described previously in the section 1, Chapter I. The cell growth was determined by dry cell weight. Lipid was extracted from the mycelium, and the total lipid (TL) production was measured by weight. The TL was fractionated into neutral lipid (NL) and polar lipid (PL) by silicic acid chromatography. Neutral lipid distribution was analyzed by TLC. Polar lipid distribution was analyzed by HPLC with internal standard as described by the authors[48]. Fatty acid composition of each lipid fraction was analyzed with gas chromatograph equipped with a flame ionization detector.

Results and Discussion

Cell growth and lipid accumulation. Time course of the cell growth and lipid accumulation at 30℃ and at 11.4 of C/N ratio using ammonium nitrate as a nitrogen source by *M. isabellina* IFO 7884 are shown in **Figure 1**. The maximum dry cell weight was obtained at 7th day of incubation period. The amount of lipid increased in parallel with the cell growth and reached

the maximum of 2.43 g/L-medium at 8th day. Lipid content in dry cell weight were around 40 % at any incubation period except the beginning.

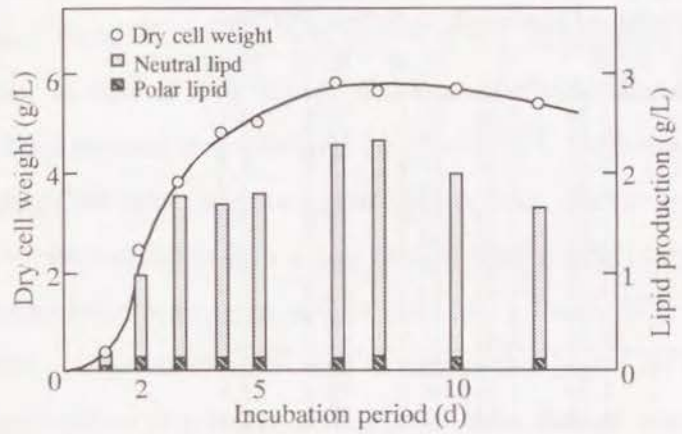


Figure 1. Growth curve and lipid production of *Mortierella isabellina* IFO 7884 grown at 30℃.

Figure 1 also shows the lipid fractions of NL and PL. Most lipid, 95% of TL was composed of NL. Polar lipid content decreased from approximately 4% at the early stage of cultivation phase to 2% at the stationary phase. Most lipid in mycelium was shown to be stored as accumulative lipid as triacylglycerol. The major fatty acid composition of NL, oleic acid of 45%, palmitic acid of 35% and linoleic acid of 7%, did not change almost through the incubation periods. Fatty acid composition of PL, on the other hand, changed greatly accompanied by cell growth, that is, linoleic acid and GLA levels decreased, from 25 to 10% and from 30 to 10%, palmitic acid and oleic acid levels increased, from 30 to 50% and from 15 to 25%, respectively.

Effects of C/N ratio and incubation temperature on lipid accumulation. The effect of incubation temperature, C/N ratio of the medium on the cell growth and lipid accumulation of the two strains of *M. isabellina* (IFO 7884 and IFO 7824) are shown in **Table 1** and **Table 2**. Period of 10 days was

chosen as a common incubation period in comparison with data of various cultural condition.

Table 1. Influence of C/N ratio and incubation temperature on cell growth and lipid formation of *Mortierella isabellina* IFO7884

C/N ratio	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
5.7	20	3.94	1.38	35.1	32.6	2.5
	30	5.58	2.02	36.2	34.1	2.1
	35	0.90	0.28	31.8	28.9	2.9
11.4	15	4.09	1.62	39.5	35.7	3.8
	20	7.30	3.12	42.8	39.8	3.0
	30	5.55	2.22	39.9	37.7	2.2
	35	0.79	0.27	34.7	32.1	2.6
114	20	2.99	1.92	64.2	62.3	1.9
	30	3.31	2.14	64.6	62.3	2.3
	35	1.58	0.81	51.1	47.4	3.7
343	20	1.42	1.19	83.5	80.1	3.4
	30	1.53	1.15	75.2	72.4	2.8
	35	1.54	1.08	70.5	67.4	3.1

Nitrogen source = Ammonium nitrate DC:Dry cell weight
TL:Total lipid production NL:Neutral lipid fraction
PL:polar lipid fraction

Table 2. Influence of C/N ratio and incubation temperature on cell growth and lipid formation of *Mortierella isabellina* IFO 7824

C/N ratio	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
5.7	20	2.92	1.06	36.3	33.8	2.5
	30	2.35	0.80	34.1	32.3	1.8
	35	0.22	-	-	-	-
11.4	15	3.01	1.17	38.8	35.4	3.4
	20	4.05	1.42	35.2	32.2	3.0
	30	4.38	2.01	45.8	43.6	2.2
	35	0.20	-	-	-	-
114	20	2.80	1.62	57.7	55.8	1.9
	30	3.34	2.05	61.4	59.3	2.1
	35	0.19	-	-	-	-
343	20	0.79	0.64	80.3	76.8	3.5
	30	1.16	0.74	63.6	60.2	3.4
	35	0.19	-	-	-	-

Nitrogen source = Ammonium nitrate DC:Dry cell weight
TL:Total lipid production NL:Neutral lipid fraction
PL:polar lipid fraction

The two strains of IFO 7884 and 7824 resulted in the highest cell growth at C/N ratio of 11.4. Cultural temperature of 30℃ mostly showed a higher cell growth except the culture of IFO 7884 at C/N ratio of 11.4. The strain of IFO 7824 appeared to have lower temperature requirement since

little growth occurred at 35℃. The lipid content did not depend on cultural temperature but was strongly influenced by C/N ratio. The highest lipid content, 83.5% was obtained at 343 C/N ratio with IFO 7884, and that more than 70% of TL content was shown at any temperature condition at the C/N ratio. In case of IFO 7824, it also obtained high value of 80.3% and 63.6% of lipid content was obtained 30℃ and 20℃, respectively. Although the higher C/N ratio gave the higher lipid content, slower growth at these higher C/N ratios resulted in a lower overall lipid productivity. The highest lipid accumulation was given at C/N ratio of 11.4 and 20℃ by the strain of IFO 7884. Chester *et al.*[6] also examined that high C/N ratio gave high lipid accumulation but lower cell growth than that of lower C/N ratio in *M. vinacea*. These result showed higher C/N ratio was not necessarily suitable condition for lipid accumulation owing to lower growth rate in spite of higher lipid content. When lipid production was aimed using *M. isabellina*, it is necessary to choose the conditions for a high lipid content as well as cell growth.

Effect of nitrogen source on lipid accumulation. The effects of various nitrogen source on cell growth and lipid accumulation of the two strains, IFO 7884 and IFO 7824 were also examined (**Table 3** and **Table 4**). Nitrate-N was not assimilated as nitrogen source by these cultures of each strains. Urea gave a higher cell growth but lower lipid production compared to ammonium sulfate in both strains. The highest cell growth reached more than 11g/L-medium when urea was used as nitrogen source at 20℃ cultivation of IFO 7884. However, the highest lipid production of 4.20 g/L-medium was observed when ammonium sulfate was used as nitrogen source at 20℃ cultivation. Ammonium sulfate shown to be the best source of nitrogen for production of lipid by these strains. Neutral lipid content is in

proportion to TL content, but PL content were a comparatively constant (2.2% to 4.4%).

Table 3. Influence of nitrogen source on cell growth and lipid formation by *Mortierella isabellina* IFO 7884

Nitrogen source	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
$(NH_2)_2CO$	20	11.20	2.82	25.2	22.1	3.1
	30	10.06	2.88	28.6	24.8	3.8
$(NH_4)_2SO_4$	20	8.83	4.20	47.6	43.2	4.4
	30	6.60	2.52	38.2	34.9	3.3
NH_4NO_3	20	7.30	3.12	42.8	39.8	3.0
	30	5.55	2.22	39.9	37.7	2.2
$NaNO_3$	20	0.22	-	-	-	-
	30	0.31	-	-	-	-
KNO_3	20	0.23	-	-	-	-
	30	0.26	-	-	-	-

C/N ratio=11.4 DC:Dry cell weight TL:Total lipid
NL:Neutral lipid PL:Polar lipid

Table 4. Influence of nitrogen source on cell growth and lipid formation by *Mortierella isabellina* IFO 7824

Nitrogen source	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
$(NH_2)_2CO$	20	9.70	1.88	19.4	15.7	3.7
	30	6.74	0.55	8.1	4.6	3.5
$(NH_4)_2SO_4$	20	5.20	1.94	37.3	34.6	2.7
	30	3.64	1.94	53.3	50.7	2.6
NH_4NO_3	20	4.05	1.42	35.2	32.2	3.0
	30	4.38	2.01	45.8	43.6	2.2
$NaNO_3$	20	0.26	-	-	-	-
	30	0.28	-	-	-	-
KNO_3	20	0.26	-	-	-	-
	30	0.28	-	-	-	-

C/N ratio=11.4 DC:Dry cell weight TL:Total lipid
NL:Neutral lipid PL:Polar lipid

Fatty acid compositions of the two strains, IFO 7884 and 7824 cultured on urea or ammonium sulfate as nitrogen source are shown in **Table 5**. The fatty acid compositions of NL did not influenced by nitrogen source so strongly as that of PL in both strains. Fatty acid compositions of PL showed the increase of GLA but decrease of palmitic acid when urea was

used as nitrogen source. Nitrogen source affected more strongly to PL as cell membrane lipid than to NL as accumulative lipid.

Table 5. Fatty acid compositions of two strains of *Mortierella isabellina* IFO 7884 and 7824 incubated with urea and ammonium sulfate as a nitrogen source

IFO No.	Nitrogen source	Lipid	Fatty acid composition (%) 14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3*	UFA (%)
7884	$(NH_2)_2CO$	NL	1.4	0.2	28.9	4.2	0.0	0.1	3.4	55.0	3.4	3.2	65.9
		PL	0.5	0.9	10.7	1.5	0.1	0.3	0.6	46.5	20.4	18.1	86.8
	$(NH_4)_2SO_4$	NL	1.5	0.1	32.6	3.2	0.0	0.2	2.9	52.2	4.2	2.7	62.5
		PL	0.8	Tr	23.1	4.2	0.0	0.4	2.2	51.6	11.2	5.8	73.2
7824	$(NH_2)_2CO$	NL	0.8	0.3	11.3	4.6	0.0	0.2	0.6	59.5	12.5	10.1	86.9
		PL	0.4	0.8	10.0	1.5	0.2	0.4	0.9	47.1	20.0	18.4	87.4
	$(NH_4)_2SO_4$	NL	0.5	0.1	17.5	1.7	0.0	0.2	2.7	57.7	11.3	8.2	81.8
		PL	0.3	0.4	14.5	1.5	0.3	0.8	1.1	37.1	32.0	11.7	83.1

* γ-Linolenic acid UFA:Total unsaturated fatty acid Tr:Trace

Changes of lipid distribution at various C/N ratio and nitrogen source. Neutral lipid distributions of the two strains of IFO 7884 and IFO 7824 at various cultural condition are shown in **Table 6** and **Table 7**. At higher temperature, decrease in TG content as well as increase in diacylglycerol and free fatty acid contents were observed compared with temperature. Content of sterol ester and free sterol became the highest value at C/N ratio of 11.4, whose condition gave the highest cell growth. Using urea as nitrogen source, increase in diacylglycerol and free sterol content as well as decrease in TG content were observed compared with ammonium nitrate as nitrogen source. Ammonium sulfate as a nitrogen source, showed a similar NL composition to that with ammonium nitrate in both strains. Content of TG resulted mostly in proportion to the NL content. However, in case of IFO 7884, TG content was lower, even though lipid content was high. The phenomenon may be influenced by other cultural conditions, which is to be solved.

Table 6. Neutral lipid distributions in *Mortierella isabellina* IFO 7884, incubated at various C/N ratio and nitrogen source

Nitrogen source	C/N ratio	Temp (℃)	Neutral lipid distribution (%) TG	1,2-DG	1,3-DG	MG	FFA	SE	FS
NH_4NO_3	5.7	20	89.1	7.3	1.6	Tr	0.7	0.5	0.8
		30	87.1	5.7	2.3	2.1	1.7	0.4	0.4
	11.4	15	95.2	2.7	Tr	Tr	Tr	0.9	1.1
		20	90.2	5.7	1.1	1.1	0.4	0.7	0.7
		30	85.3	4.8	3.3	3.5	1.4	0.8	0.6
		35	74.3	10.5	6.7	Tr	7.0	0.3	1.0
	114	20	90.2	4.6	1.7	2.1	0.5	0.3	0.4
		30	87.0	5.3	2.3	2.9	0.9	0.7	0.5
	343	20	93.8	3.2	0.8	1.3	0.2	0.3	0.5
		30	86.4	4.6	2.8	3.5	1.3	0.6	0.6
$(NH_2)_2CO$	11.4	20	84.7	7.1	1.4	2.7	0.9	0.9	2.1
		30	58.0	35.0	1.3	0.8	0.2	0.9	3.5
$(NH_4)_2SO_4$	11.4	20	75.8	17.0	1.0	1.1	3.3	0.8	0.7
		30	76.8	15.4	3.6	Tr	2.9	0.7	0.4

TG:Triacylglycerol DG:Diacylglycerol MG:Monoacylglycerol
FFA:Free fatty acid SE:Sterol ester FS:Free sterol Tr:Trace

Table 7. Neutral lipid distributions in *Mortierella isabellina* IFO 7824, incubated at various C/N ratio and nitrogen source

Nitrogen source	C/N ratio	Temp (℃)	Neutral lipid distribution (%) TG	1,2-DG	1,3-DG	MG	FFA	SE	FS
NH_4NO_3	5.7	20	91.6	4.1	3.2	0.3	0.2	Tr	0.7
		30	94.1	2.8	0.9	1.3	0.1	Tr	0.6
	11.4	20	91.6	4.3	2.5	0.7	0.1	Tr	0.8
		30	96.1	2.0	Tr	0.9	0.3	Tr	0.6
	114	20	95.7	2.6	0.8	0.4	0.1	Tr	0.4
		30	94.1	2.6	1.4	1.2	0.1	Tr	0.5
	343	20	93.5	4.1	1.8	Tr	Tr	Tr	0.6
		30	85.7	7.2	3.6	2.3	0.2	Tr	0.9
$(NH_2)_2CO$	11.4	20	56.4	28.5	1.2	2.2	7.7	0.6	3.0
		30	55.1	27.1	3.1	3.0	1.3	1.4	8.7
$(NH_4)_2SO_4$	11.4	20	93.1	2.9	0.4	1.3	0.4	1.0	0.8
		30	85.6	8.2	1.5	1.3	1.6	1.0	0.7

TG:Triacylglycerol DG:Diacylglycerol MG:Monoacylglycerol
FFA:Free fatty acid SE:Sterol ester FS:Free sterol Tr:Trace

Table 8 shows the PL distribution of the strain IFO 7824 incubated at various C/N ratio and nitrogen source. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the major substance of the PL, and the content of both lipids was over 60% of PL at various conditions. The difference of nitrogen source did not affect polar lipid distribution but C/N

ratio affected on it. As increase in C/N ratio, 11.4 to 343, PC content was decreased and PE content was increased. High value of phosphatidylinositol content was also showed at C/N ratioof 343. There were small differences of glycolipid and phosphorus containing glycolipid at various condition.

Table 8. Polar lipid distributions in *Mortierella isabellina* IFO 7824 incubated at various C/N ratio and nitrogen source

Nitrogen source	C/N ratio	Temp (℃)	Polar lipid distribution (%) PC	PE	PS	PI	LPC	LPE	p-GL	GL	TPL (%)
NH_4NO_3	11.4	20	42.2	20.2	6.8	4.4	1.5	2.4	8.8	4.4	90.7
		30	48.4	14.8	5.4	2.0	2.2	1.1	9.9	8.4	93.4
	343	20	41.9	17.0	1.2	11.4	0.0	1.1	5.6	5.3	83.5
		30	36.2	17.0	0.4	7.8	2.5	0.8	6.5	7.9	80.3
$(NH_2)_2CO$	11.4	20	42.9	21.1	7.4	2.5	3.5	2.5	4.2	2.7	86.3
		30	40.4	23.6	5.9	Tr	Tr	3.6	4.8	6.3	84.6
$(NH_4)_2SO_4$	11.4	20	41.2	21.7	2.3	5.8	1.4	1.5	8.0	5.2	87.2
		30	32.4	25.9	0.9	5.1	0.0	Tr	11.0	16.0	91.3

PC:Phosphatidylcholine PE:Phosphatidylethanolamine PS:Phosphatidylserine PI:Phosphatidylinositol LPC:Lysophosphatidylcholine LPE: Lysophosphatidylethanolamine p-GL:Phosphorus containing glycolipid GL:Glycolipid TPL:Total polar lipids detected by HPLC using internal standerd Tr:Trace

Summary

The influence of cultural conditions, especially C/N ratio of medium, cultural temperature, and nitrogen source, on the cell growth and accumulation of lipid in the mycelium of the two strains of *Mortierella isabellina* (IFO 7884 and IFO 7824) were investigated. It was found that the contents of lipid in the mycelium of the strains increased with increase of C/N ratio. The highest lipid content of 83.5% was obtained at C/N ratio of 343 at 20℃ with the strain of IFO 7884 using NH_4NO_3 as nitrogen source. The maximum accumulation of lipid, 4.20 g/L-medium, on the other hand, occurred at C/N ratio of 11.4 and 20℃ using $(NH_4)_2SO_4$. Ammonium sulfate was the best nitrogen source for production of lipid by these strains. Fatty acid composition of NL and PL were determined. Fatty acid

compositions of NL did not influenced by nitrogen source so strongly as that of PL in both strains. The influence of the cultural conditions on NL and PL distribution were also investigated. At higher temperature, decrease in TG content as well as increase in diacylglycerol and free fatty acid contents were observed.

Section 2 Influence of cultural conditions on lipid compositions of *Mortierella isabellina* using *n*-paraffin as carbon source

Some microorganisms can utilize the *n*-paraffins with carbon chain length of C5 to C19 as carbon source, as well as glucose. The author examined the lipid and γ-linolenic acid (GLA) production by various fungus using glucose as carbon source, *Mortierella isabellina* was found to accumulate a large amount of lipid intracellulaly among the members of the order Mucorales. Moreover, relatively high contents of GLA was obteined under the cultural conditions with *n*-decane as the carbon source[Chapter I, section 2].

This section deals with the investigation of the influence of cultural conditions on lipid production, lipid distribution and fatty acid composition using *n*-paraffins as carbon source by the two strains of *M. isabellina* IFO 7884 and 7824.

Materials and Methods

Microorganisms and cultural conditions. Two strains of *Mortierella isabellina* IFO 7884 and 7824 were used in this study. Basically, the liquid culture medium contained the followings; 9.1 g *n*-decane, 2.0 g KH_2PO_4, 0.91 g NH_4NO_3, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 2.0 mg Thiamin-HCl, 0.02 mg D-Biotin, and 0.2 mL Tween-20 in 1000 mL of distilled water. Initial pH of each medium were adjusted to 4.6. The C/N ratio of the medium was 24.1. Medium of the different C/N ratio were adjusted by the addition of NH_4NO_3. Nitrogen source varied by adding at 24.1 of C/N ratio instead of ammonium nitrate. The

microorganisms were grown in 1000 mL erlenmyer flasks containing 400 mL liquid medium at various temperatures under rotary shaking (150 rpm).

Analytical methods. Analytical methods were performed as described previously in the section 1 and 2 of Chapter I. The cell growth was determined by dry cell weight. Lipid was extracted from the mycelium, and the total lipid (TL) production was measured by weight. The total lipid was fractionated into neutral lipid (NL) and polar lipid (PL) by silicic acid chromatography. Neutral lipid distribution was analyzed by TLC. Polar lipid distribution was analyzed by HPLC with internal standerd. Fatty acid composition were analyzed as methyl esters with gas chromatograph equipped with a flame ionization detector.

Results and Discussion

Cell growth and lipid accumulation. Time course of the cell growth and lipid accumulation at 30℃ and 24.1 of C/N ratio using ammonium nitrate as nitrogen source by *M. isabellina* IFO 7884 are shown in **Figure 1**. The maximum dry cell weight of 2.65 g/L-medium was obtained at 13th day. The lipid content in dry cell weight increased from 12.5% at 4th day to maximum of value of 35% at 13th day, and the amount of lipid reached to 0.93 g/L-medium. The value was about one fourth of that on glucose culture. In *n*-decane culture growth rate was slow compared with glucose culture as shown in section 1 of Chapter II.

Figure 1 also shows the lipid fractions of NL and PL . The main lipid fraction in TL was NL, and it increased from 70% at 6th day to 90% at 13th day. Amount of polar lipid remained almost constant value in 75 mg/L, throughout incubation period from 6th day to 15th day. Content of polar

lipid, therefore, was decreased at the logalithmic phase to the stationary phase.

Fatty acid composition of these lipid were analyzed. Palmitic acid showed the highest content of neutral lipid at 35 to 45%, and increased at logalithmic phase, but decreased at stationary phase. Oleic acid and linoleic acid contents were almost constant in NL except 6th day. γ -Linolenic acid content was also almost constant value of 8% in NL throughout the cultivation period. Changes of fatty acid composition in PL were followings; GLA content increased from 10% (6 day) to 15% (9th day) at the logalistic phase to stationary phase, oleic acid also increased, but palmitic acid and linoleic acid decreased at same incubation period.

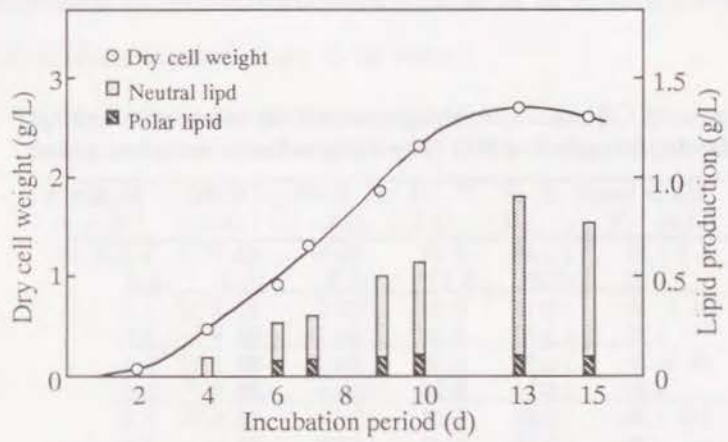


Figure 1. Growth curve and lipid production of *Mortierella isabellina* IFO 7824 grown at 30℃ using *n*-decane as carbon source.

Effects of C/N ratio and nitrogen source on lipid accumulation. The effects of C/N ratio and nitrogen source on cell growth and lipid accumulation of the two strains, IFO 7824 and IFO 7884 at 20℃ and 30℃ of cultibation temperature were examined. For the comparison of various cultural condition, 10 days was chosen for standard incubation period.

Table 1 shows the cell growth and lipid production of the strain of IFO 7824. The maximum cell growth was obtained at C/N ratio of 24.1 and 30 ℃ among the culture of various C/N ratio with ammonium nitrate. Culture at lower C/N ratio of 6.0 resulted in higher cell growth at 20℃ of lower temperature. The highest value of 937 mg/L was obtained at C/N ratio of 24.1 and 30℃, and the second highest was C/N ratio of 6.0 and 20℃. Compared with glucose as carbon source, *n*-decane culture resulted in relatively constant lipid content at various C/N ratio. Cultures using ammonium sulfate as nitrogen source gave a similar growth and lipid content to those using ammonium nitrate as nitrogen source. Urea as a nitrogen source resulted in the highest cell growth, but lipid content was low, less than 10%, compered with 20 to 30% with ammonium nitrate or ammonium sulfate.

Table 1. Influence of C/N ratio and nitrogen source on cell growth and lipid formation by *Mortierella isabellina* IFO 7824 using *n*-decane as carbon source

Nitrogen source	C/N ratio	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
NH_4NO_3	6.0	20	2.48	0.64	25.9	20.7	5.2
		30	0.89	0.15	16.9	12.4	4.5
	24.1	20	1.16	0.23	20.0	13.0	7.0
		30	2.75	0.94	34.1	30.6	3.5
	96.6	20	0.93	0.26	28.2	23.1	5.1
		30	1.14	0.36	31.1	28.0	3.1
	241	20	0.36	0.08	23.8	16.8	7.0
		30	0.49	0.12	23.5	20.0	3.5
$(NH_2)_2CO$	24.1	20	2.10	0.17	8.2	3.1	5.1
		30	3.16	0.28	9.0	4.3	4.7
$(NH_4)_2SO_4$	24.1	20	0.73	0.13	18.4	10.9	7.5
		30	2.23	0.93	41.6	35.6	6.0

DC:Dry cell weight TL:Total lipid NL:Neutral lipid PL:Polar lipid

Table 2 shows the result of the strain of IFO 7884. Cell growth and lipid content were smaller than those with the strain of IFO 7824. The highest cell growth was only 2 g/L at C/N ratio of 24.1 and 30℃, and the highest lipid production was only 336 mg/L at C/N ratio of 96.6 and 30℃.

These results were compared with the glucose culture of previous section. The most prominant difference was IFO 7884 showed a higher cell growth and lipid accumulation than IFO 7824 with glucose culture, but *vice versa* with *n*-decane culture. Furthermore, *n*-decane culture did not result in so much lipid accumulation as glucose culture at high C/N ratio (80%-lipid content). *n*-Decane seems to have some limitation in terms of carbon source for lipid accumulation. Polar lipid content with *n*-decane were approximately 2 times higher than that with glucose. Urea as nitrogen source resulted in high cell growth but low lipid content with both *n*-decane and glucose. These results seemed to come not only from the different dependency of the two strains on nitrogen source, but also different pH value during there culture period. Effects of cultivation pH on cell growth and lipid accumulation are to be solved.

Table 2. Influence of C/N ratio and nitrogen source on cell growth and lipid formation by *Mortierella isabellina* IFO 7884 using *n*-decane as carbon source

Nitrogen source	C/N ratio	Temp. (℃)	DC (g/L)	TL (g/L)	TL/DC (%)	NL/DC (%)	PL/DC (%)
NH_4NO_3	6.0	20	0.71	0.10	13.4	7.2	6.2
		30	0.50	0.11	23.0	17.8	5.2
	24.1	20	1.65	0.15	9.0	6.1	2.9
		30	1.97	0.23	11.8	7.0	4.8
	96.6	20	0.94	0.23	24.6	19.9	4.7
		30	1.18	0.34	28.5	23.9	4.6
	241	20	0.40	0.12	29.1	23.3	5.8
		30	0.64	0.19	29.5	23.4	6.1
$(NH_2)_2CO$	24.1	20	1.80	0.24	13.1	7.7	5.4
		30	0.98	0.13	12.8	4.8	8.0
$(NH_4)_2SO_4$	24.1	20	1.21	0.17	14.1	8.8	5.3
		30	1.43	0.14	9.8	5.1	4.7

DC:Dry cell weight TL:Total lipid NL:Neutral lipid PL:Polar lipid

Change of lipid and fatty acid compositions at various C/N ratio and nitrogen source. Neutral lipid distribution of the strain of IFO 7824 incubated at various C/N ratio and nitrogen source is shown in **Table 3**. Triacylglycerol content changed from 40 to 90%, in accordance with neutral

lipid content. The culture of high lipid content using ammonium nitrate at C/N ratio of 24.1 and 30℃ gave high TG content of 88.7%, and the culture of lower lipid content with urea gave lower TG content. In the case of urea, it showed high level of free sterol content. In termes of the content of free sterol, however, similar values were obtained among all cultures.

Table 3. Neutral lipid distributions in *Mortierella isabellina* IFO 7824 incubated at various C/N ratio and nitrogen source using *n*-decane as carbon source

Nitrogen suorce	C/N ratio	Temp (℃)	Neutral lipid distribution (%) TG	1,2-DG	1,3-DG	MG	FFA	SE	FS
NH_4NO_3	6.0	20	83.1	10.4	1.3	0.8	0.5	1.6	2.1
		30	76.5	16.9	1.3	0.8	0.5	0.9	3.1
	24.1	20	57.0	9.2	7.4	0.7	19.5	0.3	5.6
		30	88.7	3.2	1.4	0.7	3.0	0.2	2.7
	96.6	20	84.6	8.4	1.7	0.6	1.2	1.0	2.3
		30	78.4	14.4	1.7	1.1	1.5	0.3	2.2
	241	20	77.5	12.7	1.8	1.7	2.0	0.5	3.5
		30	71.8	20.2	2.6	1.0	1.1	0.6	2.6
$(NH_2)_2CO$	24.1	20	38.6	9.1	7.2	16.4	8.8	0.2	18.6
		30	58.1	4.8	10.6	4.6	5.9	0.3	15.1
$(NH_4)_2SO_4$	24.1	20	59.3	7.2	4.2	5.8	17.0	0.2	5.9
		30	88.3	0.9	4.2	0.9	2.1	0.3	3.2

TG:Triacylglycerol DG:Diacylglycerol MG:Monoacylglycerol FFA:Free fatty acid SE:Sterol ester FS:Free sterol Tr:Trace

Table 4. Polar lipid distributions in *Mortierella isabellina* IFO 7824 incubated at various C/N ratio using *n*-decane as carbon source

C/N ratio	Temp (℃)	Polar lipid distribution (%) PC	PE	PS	PI	LPC	LPE	p-GL	GL	TPL (%)
11.4	20	37.5	8.9	5.1	8.1	3.0	2.2	6.5	8.7	80.0
	30	52.2	13.4	1.1	7.0	1.1	0.9	7.6	16.5	99.8
24.1	20	44.7	14.0	0.2	4.3	Tr	1.4	8.2	8.1	80.9
	30	41.7	6.9	3.4	7.6	0.0	Tr	12.9	15.3	87.8
96.6	20	43.5	8.9	0.4	4.6	Tr	1.0	8.2	7.0	73.2
	30	46.8	9.8	2.6	8.0	Tr	Tr	8.7	22.0	97.9
241	20	43.7	14.3	3.7	9.1	0.0	0.7	8.3	7.8	87.6
	30	32.9	7.2	4.0	11.2	0.0	Tr	13.7	13.4	82.4

PC:Phosphatidylcholine PE:Phosphatidylethanolamine PS:Phosphatidylserine PI:Phosphatidylinositol LPC:Lysophosphatidylcholine LPE:Lysophosphatidylethanolamine p-GL:Phosphorus containing glycolipid GL:Glycolipid TPL:Total polar lipids detected by HPLC using internal standerd Tr:Trace

Polar lipid distribution of the strain of IFO 7824 incubated at various C/N ratio is shown in **Table 4**. At any condition, phosphatyiylcholine and phosphatidylethanolamin were also the principle constituent. Smaller influences on PL distribution by the difference of C/N ratio was obserbed. Cultivation at 30℃ gave higher value of phosphorus glycolipid and glycolipid content than those at 20℃.

Table 5. Influence of carbon number of *n*-paraffin on cell growth and fatty acid composition of *Mortierella isabellina* IFO 7824 and 7884 incubated with ammonium nitrate as nitrogen source at C/N ratio of 24.1

IFO No.	Paraffin	DC (g/L)	TL (mg/L)	TL/DC (%)	Fatty acid composition (%) 10:0	12:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3*
7824	Octane	0.04	-	-												
	Decane	2.75	938	34.1	0.1	0.1	1.8	1.1	41.2	3.4	1.4	1.7	3.4	27.1	10.3	7.8
	Dodecane	0.32	61	18.9	0.3	2.2	3.5	2.5	15.0	7.5	1.6	1.5	3.0	35.4	4.6	15.3
	Tetradecane	0.04	-	-												
	Hexadecane	0.04	-	-												
7884	Octane	0.05	-	-												
	Decane	1.97	232	11.8	0.0	0.0	3.2	0.8	43.0	2.7	0.2	0.0	2.9	21.2	6.0	18.9
	Dodecane	0.20	42	21.4	2.0	0.6	0.5	1.2	14.0	4.0	3.4	4.4	2.9	29.8	26.7	4.2
	Tetradecane	0.16	8	4.6	0.0	0.2	11.1	0.9	12.4	5.4	0.8	0.6	2.2	39.6	8.6	14.9
	Hexadecane	0.20	13	6.7	0.0	0.0	1.9	1.2	18.9	5.0	1.3	2.9	2.7	40.2	8.8	15.1

* γ-Linolenic acid UFA:Total unsaturated fatty acid Tr:Trace

Influence of carbon number of n-paraffin on cell growth, lipid production and fatty acid composition. **Table 5** shows the influence of carbon number of *n*-paraffins on cell growth, lipid accumulation and fatty acid composition of the two strains IFO 7824 and IFO 7884. Their ability to consume of *n*-paraffins was quite limited in terms of carbon number. *n*-Decane supported the maximum cell growth among *n*-octane to *n*-hexadecane. The strain of IFO 7824 were able to use only *n*-dodecane, except *n*-decane. In case of strain of IFO 7884, it was able to use *n*-dodecane to *n*-hexadecane, though their usability was only 10% of *n*-decane. When *n*-tetradecane and *n*-hexadecane were used, mirystic acid and palmitic acid contents were increased, respectivery. which was indicating that both

carbon source were taken in and transformed to fatty acid directly. As for GLA content of TL on *n*-decane 7.8 and 18.9% with IFO 7824 and IFO 7884, respectively, were obtained. These value were reratively higher than those on glucose culture.

Summary

Using *n*-paraffine as carbon source, influence of cultural conditions, especially C/N ratio of medium, growth temperature, nitrogen source, and chain length of *n*-paraffins on the cell growth and lipid production in the two strains of *M. isabellina*, IFO 7884 and IFO 7824 were investigated. *n*-Decane supported the maximum cell growth among *n*-octane to *n*-hexadecane. The maximum accumulation of lipids, 937 mg/L-medium, was obtained with the strain of IFO 7824 at C/N ratio of 24.1 and 30℃ using ammonium nitrate as nitrogen source. *n*-Decane culture did not result in so much lipid accumulation as glucose culture at high C/N ratio(80%-lipid content). It resulted in relatively constant lipid content at various C/N ratio. *n*-Decane seems to have some limitation in terms of carbon source for lipid accumulation. Polar lipid content were approximately 2 times higher than glucose culture.

Section 3 Influence of cultural conditions on lipid productivity of *Mortierella isabellina*

The cultivation factors and medium composition affecting the cell growth and lipid accumulation. It is well known that C/N ratio (ratio of carbon to nitrogen source) of culture medium affects greatly on a lipid content in cell mass[49]. In the study of oleaginous yeasts, *Candida* and *Rhodotorula*, quantity of lipid accumulated was increased by restricting a nitrogen source[50-53]. Varying pH also might play a role in altering lipid accumulation and lipid composition in oleaginous yeast[54]. The author searched for fungi which have high lipid productivity for the purpose of lipid production by microorganism, and found that the strain of *Mortierella isabellina* had not only high lipid productivity but some amount of γ-linolenic acid (GLA) as shown in the previous chapter. Furthermore, influence of cultural conditions, especially nitrogen source, C/N ratio, and culture temperature, on cell growth, lipid accumulation, and lipid composition in flask culture were investigated[Chapter II, section 2]. In *M. isabellina* also, lipid content of mycerium varied extremely from 30% to 80% according to C/N ratio of culture medium. Though, higher C/N ratio gave higher lipid content, it gave lower cell growth and consequently lower overall lipid production. We tried to realize both high cell growth and high lipid content simultaneously for a efficient lipid production. But the results above required more detailed examination.

This section deals with the investigation of the influence of cultural conditions, especially C/N ratio, culture pH, and temperature on cell growth and lipid accumulation of *M. isabellina* using stirred tank fermenter.

Materials and Methods

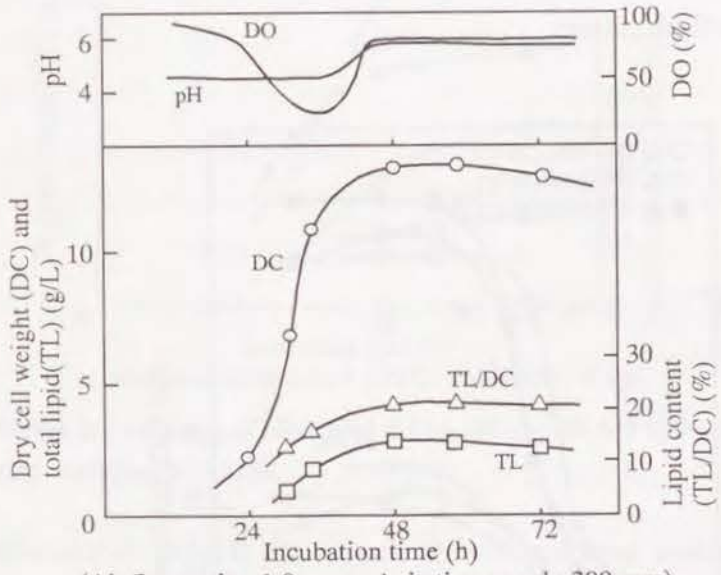
Microorganisms and cultural conditions Three strains of *M. isabellina*, IFO 7884, IFO 8308, and IFO 8183 were used in this study. Basically, the liquid culture medium contained the followings; 25 g glucose, 3.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.2 g yeast extract, 0.2 g malt extract, 0.1 g pepton, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$ and 1.0 mg $MnSO_4 \cdot 4H_2O$ in 1000 mL of distilled water. Initial pH of medium was adjusted to 4.6 except the experiment on effect of pH. The C/N ratio of this medium was 15.7, and the various C/N ratio of 7.9, 31.4 47.1 and 62.9, respectively, were adjusted by the adding 6.0 g 1.5 g, 1.0 g and 0.75 g of ammonium sulfate.

A 10 L stirred tank fermenter (Kanto-rikaki Seisakusyo, model CRUD Ⅱ) system was employed in this study. The operation conditions were; 6 L working volume, 300 rpm agitation speed, and 1.0 vvm air flow rate. The cultural pH was controlled at 4.5 with 2N NaOH except the experiment on effect of pH. Inoculum was grown in 1000 mL erlenmyer flasks containing 400 mL liquid medium under rotary shaking (150 rpm) for 3 days, and 50 mL culture broth of which was transformed to the fermenter. Samples of 100 mL were taken out for analysis at various cultivation time.

Analytical methods. Analytical methods were performed as described previously in the section 1, Chapter I. The cell growth was determined by dry cell weigh. Lipid was extracted from the mycelium, and amount of total lipid (TL, g/L-medium) was measured by weight. Fatty acid composition of TL was analyzed as methyl esters with gas chromatograph equipped with a flame ionization detector. Lipid coefficient, lipid produced from 100 g of glucose, was also used to compare the lipid productivity[55].

Results and Discussion

Time course of M. isabellina in the basal conditions. A time course of the cultivation of *M. isabellina* IFO 7884 using the basal medium and temperature at 30 ℃ is shown in **Figure 1**. The pH was maintained at 4.5 with alkali addition until nearly the end of the logarithmic growth phase and then allowed to increase to 5.5 without control. Level of dissolved oxygen was also decreased at logarithmic growth phase. At 48 h, dry cell weight reached 13 g/L, which was 30% higher than those in flask cultures shown in the section 1, Chapter II. Lipid content, on the other hand, remained relatively low (15% of dry cell weight).



(Air flow ratio : 1.0 vvm. Agitation speed : 300 rpm)

Figure 1. Growth curve of *Mortierella isabellina* IFO 7884 grown at 30℃.

Culture of IFO 7884 at 20 ℃ and two other strains of *M. isabellina* (IFO 8308 and IFO 8183) grown under the same conditions are shown in **Figure 2**. Two strains of IFO 8308 and IFO 8183 also reached more than 10 g/L of dry cell weight at 48 h. Lipid contents of these culture were 23%

and 21%, respectively, similar to that of IFO 7884 shown in **Figure 1**, and those value were lower than those in flask culture. At lower temperature of 20 ℃ culture of IFO 7884 resulted in less cell growth but higher lipid content of 26%. When we compare those results with those in flask[section 1, Chapter II], largest difference was observed in lipid content. In flask culture at C/N ratio of 11.4, more than 40% of lipid content was obtained. These values were enough high to compensate their low growth and resulted in higher lipid production than those of the fermenter. Though C/N ratio were not the same in flask and fermenter, it was thought that influence of C/N ratio were small. A change of pH during the culture period, therefore, seemed to influence lipid content greatly, because pH was lowered to around 2 in the flask culture.

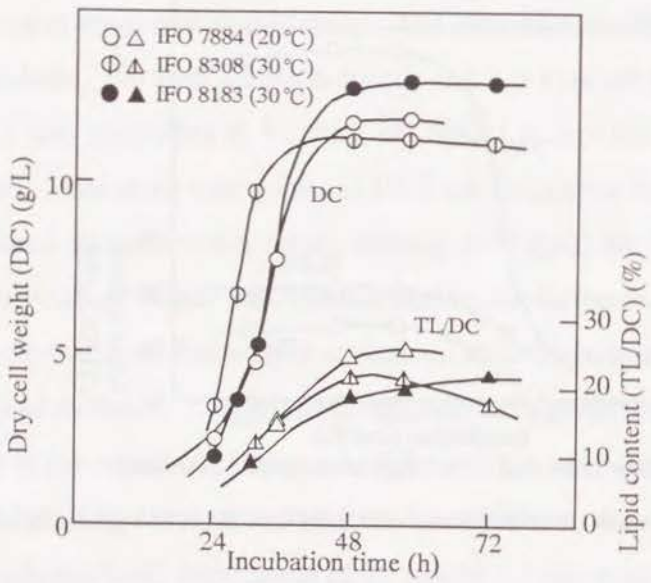
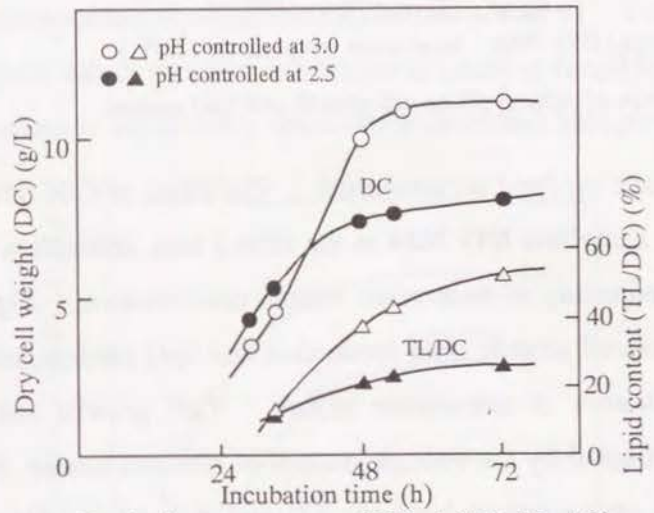


Figure 2. Growth curve and lipid content of *M. isabellina*, 3strains.

Influence of cultural pH on lipid accumulation. The influence of cultural pH on cell growth and lipid accumulation of the strain IFO 7884

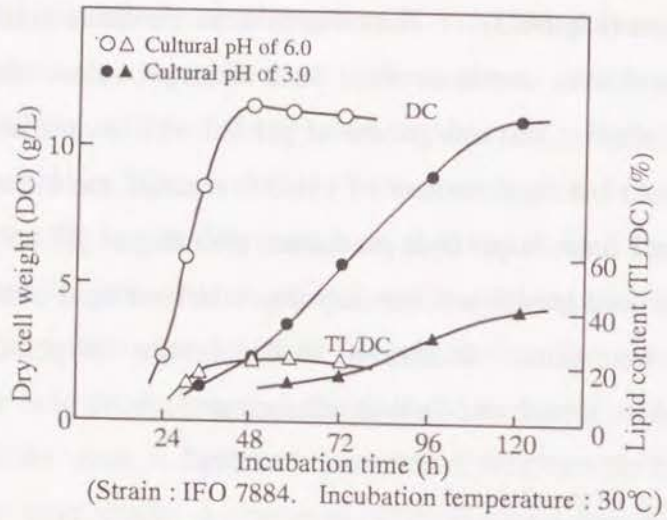
was then examined (**Figure 3**). The cultures were started at initial pH of 4.6 and pH control were started at about 30 h when pH values reached to 2.5 or 3.0 using alkali. The cell growth of pH 3.0 was lower than that of pH 4.5 (**Figure** 1) but lipid content of pH 3.0 reached more than 50%, which resulted in 2 times larger lipid production than that of pH 4.5. In the culture of pH 2.5, cell growth was not only depressed but lipid content also remained at a low value. It showed that condition of pH 2.5 was unfavorable for both growth and lipid accumulation.



(Incubation temperature : 30℃. Initial pH : 4.6.)

Figure 3. Influence of controlled pH on cell growth and lipid content of *M. isabellina* IFO 7884.

Influence of cultural pH on cell growth and lipid content is shown in **Figure 4**. When the pH was controlled from the beginning at 6.0 showed almost the same growth curve and lipid content as those of pH 4.5 (**Figure 1**). Although pH 3.0 resulted in a decrease in growth rate, the lipid content was nearly doubled to that of the pH 6.0. These results showed that a cultural condition at lower pH ranged from 3.0 to 4.5 promoted lipid accumulation.



(Strain : IFO 7884. Incubation temperature : 30℃)

Figure 4. Influence of cultural pH on cell growth and lipid content.

Effect of C/N ratio on lipid accumulation. The effect of C/N ratio was examined with *M. isabellina* IFO 7884 in the stirred tank fermenters at pH 4.5 and 30 ℃ by adjusting of ammonium sulfate concentration. **Figure 5** showed the effect of cell growth, lipid production and lipid content, cultured at various concentration of ammonium sulfate. Cell growth and lipid content were not affected by the enough amount of nitrogen source, that is, ammonium sulfate concentration between 3.0 and 6.0 g/L. However, ammonium sulfate concentration affected greatly on cell growth and lipid content at lower concentration. The highest lipid production of 5.5 g/L and lipid content of 67% in dry cell weight were given with the ammonium sulfate concentration at 0.75 g/L. In this ammonium sulfate concentration, lipid production was two times lager than that of ammonium sulfate concentration at 3.0 g/L, but it resulted in the lowest dry cell weight among the conditions examined. In ammonium sulfate concentration range from 0.75 to 3.0 g/L, lipid production decreased and dry cell weight increased with an increase of ammonium sulfate concentration. These results showed

the strain has the wide range of lipid content from 20 to 70% according the nitrogen source concentration. In flask culture, 50% of lipid content was obtained at 15 of C/N ratio as shown in section 1, Chapter II. In the present result, however, 40 to 50% of lipid content were given at C/N ratio of 30 to 50. It was thought that the difference of C/N ratios which gave a lipid content around 50% between flask culture and fermenter culture was derived from difference of pH profiles in both culture.

The highest lipid coefficient of 2.2 was obtained at 0.75 g/L of ammonium sulfate concentration (C/N ratio of 62.9). This value exceeded the highest values reported in oleaginous yeast or fungi[56,57], and that this fungal strain is a promising resource for microbial lipid production.

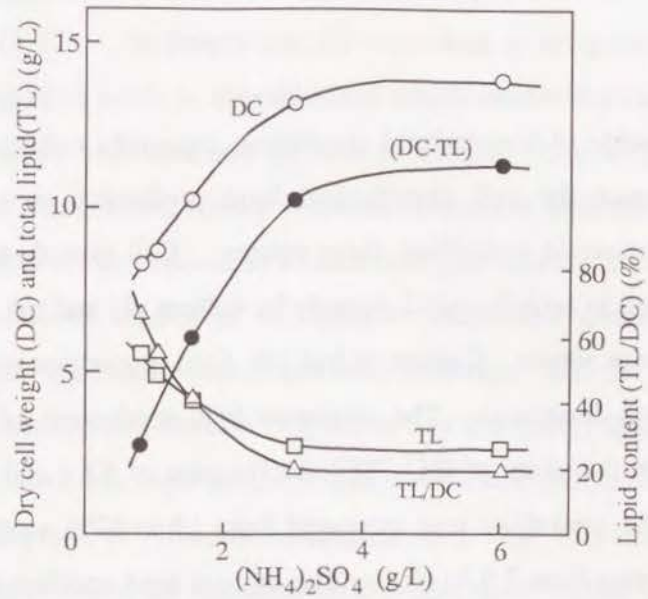


Figure 5. Influence of ammonium sulfate concentration on cell growth and lipid content.

Change of fatty acid composition at various condition. Fatty acid composition of TL harvested at stationary phase in each culture was determined. The composition of the principal fatty acids is shown in **Table**

1. Compared with the fatty acid composition with the culture at higher pH, culture at lower pH gave higher content of saturated fatty acid and lower content of oleic acid and GLA. Culture at lower nitrogen concentration gave less GLA content than that at higher nitrogen concentration.

Table 1. Influence of various cultural conditions on fatty acid composition of *M. isabellina* IFO 7884

Cultural condition			Fatty acid composition (%)						UFA
AS (g/L)	pH Int	Cont	16:0	16:1	18:0	18:1	18:2	18:3*	(%)
3.0	4.6	4.5	20.4	3.5	2.6	55.5	10.9	5.7	75.8
3.0	4.5	3.0	27.0	4.7	1.8	47.5	15.3	2.2	70.0
3.0	4.5	2.5	25.5	3.5	2.9	51.4	12.1	3.7	70.7
3.0	6.0	6.0	22.2	3.1	3.0	52.0	12.6	5.5	73.9
3.0	3.0	3.0	31.0	4.8	3.0	47.3	10.2	1.4	63.9
0.75	4.6	4.5	29.5	3.8	2.4	45.6	13.5	3.9	67.0
6.0	4.6	4.5	23.1	3.1	3.0	50.7	12.5	6.6	73.0

AS: Ammonium sulfate as nitrogen source Int: Initial pH Cont: Controlled pH UFA: Total unsaturated fatty acid * γ-Linoleni

Summary

A study was made of how cultural conditions, especially culture pH and C/N ratio, influence the cell growth and lipid production in a stirred fermenter of *Mortierella isabellina*, three strains. Cell growth and lipid content were found to be influenced strongly by culture pH and the quantity of available nitrogen source. Culture of low pH, lipid production increased, but the cell growth decreased. The maximum lipid production of 5.5 g/L was obtained with the strain of IFO 7884 at C/N ratio of 62.9 and pH 4.5. Lipid content in *M. isabellina* was increased from 19 to 67% according to increase in C/N ratio from 7.9 to 62.9. The highest lipid coefficient of 2.2 was obtained at C/N ratio of 62.9. This value exceeded the highest values reported in oleaginous yeast or fungi, and that this fungal strain is a promising resource for microbial lipid production.

Section 4 Effect of cultural conditions on lipid productivity of *Mortierella isabellina* with a culture at high cell mass

In order to realize the efficient lipid production by microorganisms, selection of a suitable strain which is capable of lipid accumulation in its cell is most important. The optimum culture conditions for lipid production should be next performed. Two important criteria for the experiment were higher cell mass per unit volume of the culture broth and higher lipid content in the cell. Higher concentration of glucose in the culture medium was a direct way to obtain higher cell mass in unit volume of the culture broth. Concentration range from 20 to 60 g/L of glucose, however, has been commonly used owing to inhibition of higher glucose concentration to fungal growth[16,30]. In fungal culture, according to its growth phase, hyphal stretching often leads to entanglement which causes increase in viscosity of culture broth and decrease in rate of oxygen transport[58]. Therefore, higher cell mass in the culture is difficult to realize, and there have been very few reports which succeed in a culture at high concentration of glucose[59]. In the previous section of this chapter, the author described the cultural conditions, especially culture pH and C/N ratio for the purpose of lipid production with *Mortierella isabellina*. In the study, it was found that the *M. isabellina* has high level of lipid coefficient defined as gram of lipid produced/gram of glucose consumed, and that this fungal strain is a promising resource for microbial lipid production[Chapter II, section 3].

This section deals with the investigation of the influence of cultural conditions on cell growth and lipid accumulation at a high concentration of glucose and molasses in the medium in order to increase in the lipid production of *M. isabellina* IFO 7884.

Materials and Methods

Cultural conditions. The medium used for flask culture contained the followings; 30 g glucose, 0.7 g urea, 1.5 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.2 g yeast extract, 0.2 g malt extract, 0.1 g pepton, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnSO_4 \cdot 4H_2O$ and 1000 mL of distilled water (C/N ratio of the medium was 40). In case of the cultures changing glucose concentration, concentration of the other components were changed to have the same ratio to that of glucose. The medium various phosphate concentration was adjusted by the amount of KH_2PO_4 added.

The medium used for stirred tank fermenter, which was basically determined by the optimization from flask culture, contained the followings; 200 g glucose, 3.7 g urea, 4.5 g $(NH_4)_2SO_4$, 5.0g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.3 g NaCl, 0.6 g yeast extract, 0.6 g malt extract, 0.3 g pepton, 30 mg $FeSO_4 \cdot 7H_2O$, 30 mg $CaCl_2 \cdot 2H_2O$, 0.6 mg $CuSO_4 \cdot 5H_2O$, 3.0 mg $ZnSO_4 \cdot 7H_2O$, 3.0 mg $MnSO_4 \cdot 4H_2O$ and 1000 mL of distilled water (C/N ratio of the medium was 40). The various C/N ratio ranged 10 to 40 was adjusted by the amount of urea added. In case of the cultures changing glucose concentration, also, concentration of the other components were changed to have the same ratio to that of glucose. The nitrogen sources were sterilized separately with other components. In case of the cultures using molasses instead of glucose, heat sterilization of medium was done under the condition of pH 3.0 in order to promote hydrolysis of sucrose.

The medium optimization were performed using 1000 mL erlenmyer flasks containing 300 mL liquid medium at 30℃ under rotary shaking at 180 rpm. A 30 L stirred tank fermenter system (Marubishi Rikasouchi, model MSJ-U2) was also employed in this study. The operation conditions were; 20 L working volume, 400-500 rpm stirrer agitation speed, and 1.0 vvm air

flow rate. The cultural pH was controlled at 4.0 with 2N NaOH. Culture was stopped when glucose concentration became zero.

Analytical method. Glucose concentration in culture broth was analyzed using a glucose analyzer (Yellow spring Instrument Co. Ltd., model 27). In the culture using molasses as a carbon source, sugar concentration in culture broth was analyzed using HPLC equipped with refractive index detector (Shimadzu, model LC-3A and RID-2A). The chromatography was performed with Unisil Q NH2 column (4.0 × 250 mm) using acetone/water (7:3 by vol.) as eluent.

The other analytical methods were performed as described previously in the section 1, Chapter I. The cell growth was determined by dry cell weight. Lipid was extracted from the mycelium, and amount of total lipid (TL, g/L-medium) was measured by weight. Fatty acid composition of the lipid was analyzed as methyl esters with gas chromatograph equipped with a flame ionization detector.

Lipid coefficient, gram of lipid produced from 100 g of glucose, and lipid productivity, gram of lipid produced/incubation time, were also used to compare the efficiency of lipid production[55].

Results and Discussion

Production of high cell mass in flask culture. In order to increase the cell growth and lipid production, high concentration of glucose in the range of 30 to 420 g/L were examined. In this experiment, urea was used for nitrogen source instead of ammonium sulfate used in the previous study in order to avoid decreased of pH. **Figure 1** shows the influence of initial glucose concentration on cell growth and lipid content. The maximum cell growth and lipid production were obtained at 180 g/L of glucose concentration. In spite of the same C/N ratio, lipid content increased from 35% to 45% in the

culture at 30 to 180 g/L of glucose concentration . Cell growth and lipid production decreased at higher glucose concentration than 200 g/L. Though growth of cell was still observed at glucose concentration higher than 300 g/L, no growth was observed at 420 g/L of glucose concentration. These results showed that *M. isabellina* is extremely tolerant to high concentration of glucose. No reports have been published on fungal culture which obtained enough cell growth and lipid production in the culture using glucose at higher concentration than 100 g/L.

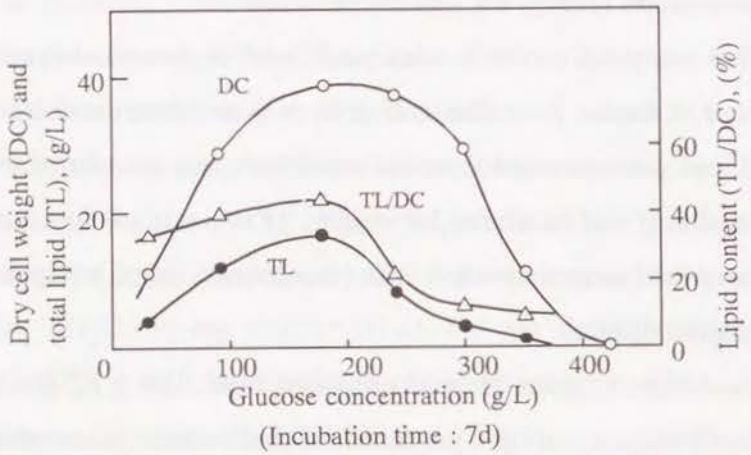


Figure 1. Influence of glucose concentration on cell growth and lipid production in flask culture.

Effect of phosphate (KH_2PO_4) concentration was examined using a medium at 180 g/L of glucose concentration (**Figure 2**). Little change of cell growth and lipid production were observed in the range of about 5 to 10 g/L. Phosphate concentration of 5.0 g, a half concentration of the basal medium was proved to be enough. Subsequent experiment culture in the stirred tank fermenter, therefore, was performed at 5.0 g/L of KH_2PO_4 with 200 g/L of glucose, and the other minor component of the medium were also lessened to a half concentration of the basal medium.

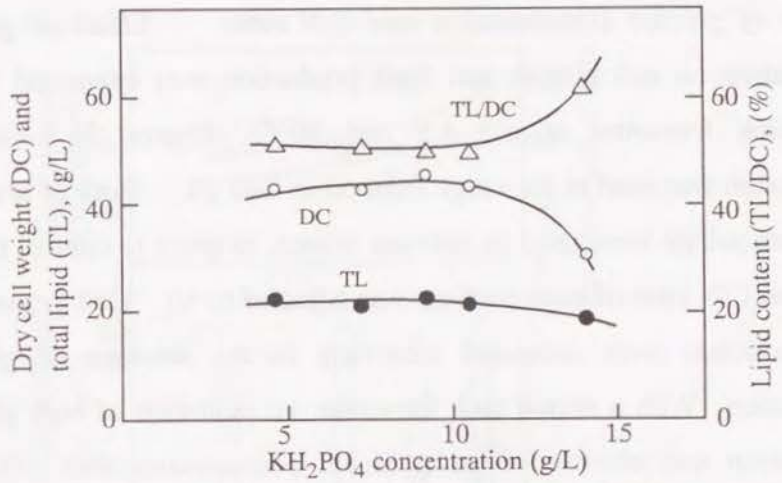


Figure 2. Effect of phosphate source concentration on cell growth and lipid production in flask culture.

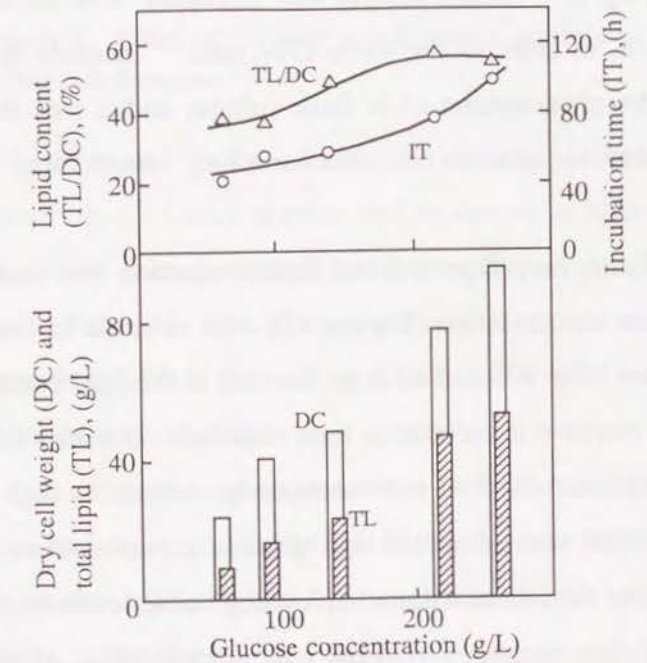


Figure 3. Effect of glucose concentration on cell growth and lipid production in stirred tank fermenter

Effect of glucose concentration and C/N ratio. Effect of glucose concentration on cell growth and lipid production was examined in the stirred tank fermenter at pH 4.5 and 30 ℃ (**Figure 3**). Glucose concentration was used in the range from 60 to 240 g/L. Both of urea and ammonium sulfate were used as nitrogen source, in order to control the pH easily, and C/N ratio of each medium was adjusted to 40. Cell growth and lipid production were increased according to the increase of glucose concentration. With a stirred tank fermenter, no inhibition of high glucose concentration was observed even at higher concentration than 200 g/L. Longer incubation time was required according to increase in initial glucose concentration. Compared with flask culture, the glucose concentrations that gave the highest cell growth and lipid production in stirred tank fermenter were in excess of 200 g/L. Lipid content also increased with increase in glucose concentration, in spite of the same C/N ratio. Increase in lipid content was the same phenomenon of in flask culture, and it was thought that high concentration glucose in the medium accelerated lipid accumulation.

The effect of C/N ratio on cell growth and lipid production was examined with 220 g/L glucose concentration (**Figure 4**). An increase in the C/N ratio in the range from 10 to 40 resulted in an increase in the lipid content 35 to 55%, but also an increase in incubation time required. It seems that the culture at high lipid content dose not necessarily obtain a high lipid productivity. The lipid content under high glucose concentrations were about 10 to 15% higher than those cultured at lower glucose levels as shown in the section 3 of this chapter. Taking into consideration of shorter incubation time, C/N ratio of 20 seems to be optimum for high lipid productivity.

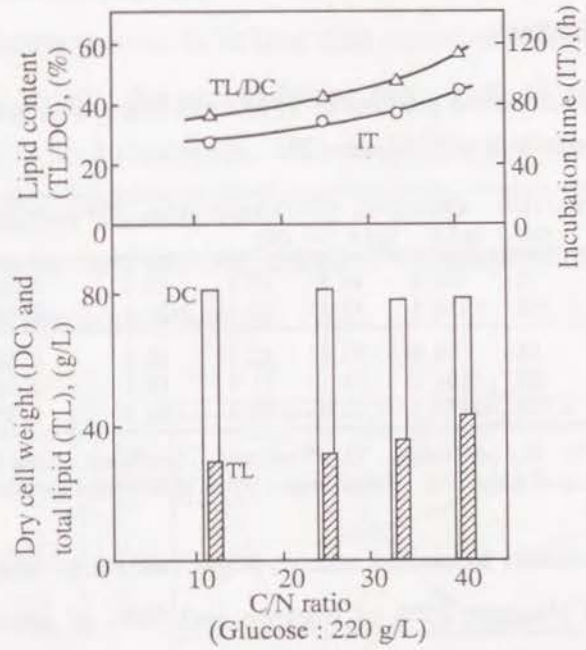


Figure 4. Effect of C/N ratio on cell growth and lipid production in stirred tank fermenter.

Cell growth and lipid productivity in the medium at high carbon source concentration. Using glucose and molasses at higher concentration as carbon source than previously, cell growth and lipid productivity were examined (**Table 1**). The glucose and molasses concentration were used in the range from 270 to 390 g/L and 180 to 300 g/L, respectively, but C/N ratio were controlled at 20.

At a very high glucose concentration (390 g/L), maximum cell growth of 156 g/L and lipid production of 83.1 g/L were obtained at 168 h of incubation time. The highest lipid coefficient of 21.3 was also obtained in the culture. The highest lipid productivity, however, was found to be 0.69 g/L/h in culture with a glucose concentration at 270 g/L. The maximum value of lipid productivity, 0.69 was more than 10 times higher than that in a

oleaginous yeast *Rodotorula*[51] or in *M. isabellina*, shown in perviously in the section 3 of this Chapter.

Table 1. Lipid production and productivity by *M. isabellina* grown in high concentration of carbon source (C/N ratio : 20)

Carbon source	Concent-ration (g/L)	IT (h)	DC (g/L)	TL (g/L)	TL/DC (%)	Coefficient	Productivity
Glucose	270	72	103.5	49.4	47.7	18.3	0.69
	390	168	156.4	83.1	53.1	21.3	0.50
Molasses	180	68	59.9	25.6	42.7	15.5	0.38
	280	108	104.2	53.1	51.0	19.0	0.49
	300	128	113.3	47.6	42.0	15.9	0.37

IT : Incubation time, DC : Dry cell weight, TL : Total lipid, Coefficient : Total lipid weight produced / 100 g of sugar (%), Productivity : Total lipid weight produced / incubation time (g/L/h)

In the culture of molasses as carbon source, sugar content of which was approximately 20% of glucose, 20% of fructose and 60% of sucrose, the strain showed diauxic growth with glucose and fructose, but very little growth with sucrose. After sucrose was hydrolyzed during heat sterilization of the medium at lower pH, molasses as carbon source indicated a similar growth to those of glucose. Though a little longer incubation time was needed, cell growth reached more than 100 g/L and lipid production around 50 g/L. These result showed that molasses was also applicable as carbon source, an increased economic feasibility of this method.

Figure 5 shows the time course of cultivation with 270 g/L glucose medium. Agitation speed was raised to 500 rpm at 24 h into the operation in order to increase oxygen transport. Large increase in dry cell weight and large amount of glucose consumption were observed at the incubation time from 24 h to 48 h. At 72 h, residual quantity of glucose became zero and dry cell weight exceeded 100 g/L. The value of dissolved oxygen and pH were lowered with increase in dry cell weight, during the incubation time from beginning to around 48 h. During the same period, the value of cell

weight without lipid, (DC-TL), was increased. The period of lipid accumulation seemed to be later than period of cell growth. An increase in dry cell weight, during incubation time from 48 to 72 h, derived from increase in lipid production. It was thought that very high lipid content of this fungus was owing to the capacity of consuming glucose and accumulating lipid after the complete consumption of nitrogen source in the late logarithmic growth phase.

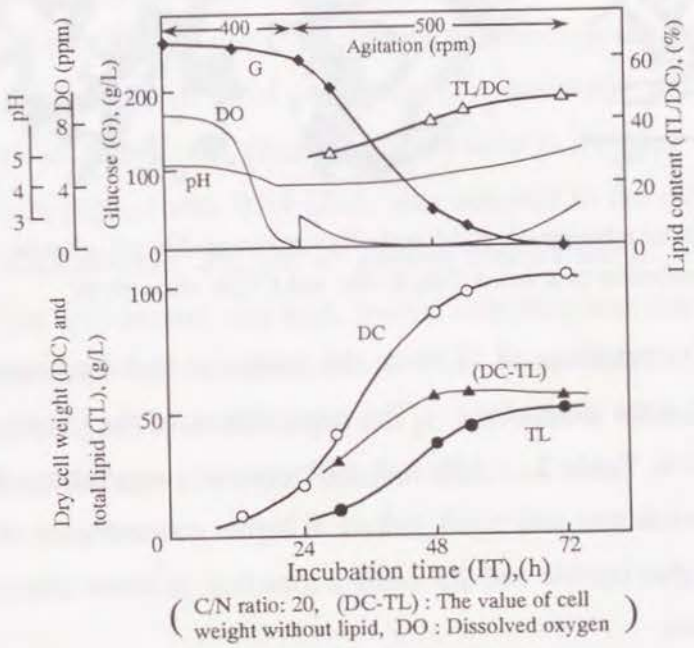


Figure 5. Time course of cultivation with 270 g/L glucose medium in stirred tank fermenter.

Figure 6 shows the microphotograph of *M. isabellina* grown on 270 g/L glucose medium at incubation times of 24, 48, 72h. The shape of the cells becomes round after the late logarithmic growth phase, from 48 to 72h. At the growth phase when lipid content was high, hyphal stretching was not observed and the fungal shape became yeast-like. This shape was considered to be beneficial to an increase in a fungal cell growth without

increase in viscosity and without decrease of oxygen transport in culture broth.

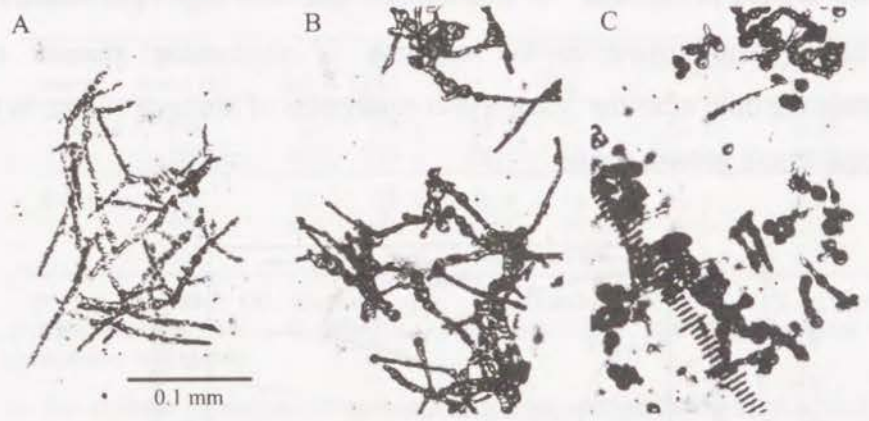


Figure 6. Microphotograph of *M. isabellina* grown on 270 g/L glucose medium. Incubation time was A:24h, B:48h, and C:72h, respectively.

Fatty acid composition of TL from the culture at high concentration of carbon source were determined. The composition of the principal fatty acids is shown in **Table 2**. Although difference of compositions between each cultural conditions was small, culture at higher concentration of carbon source gave higher linoleic and GLA content than that at lower concentration of carbon source.

Table 2. Fatty acid composition of the lipid cultured at high concentration of carbon source.

Carbon source		Fatty acid composition (%)						UFA	DU
	(g/L)	16:0	16:1	18:0	18:1	18:2	18:3*	(%)	
Glucose	270	30.8	4.7	2.7	46.1	11.7	2.5	72.5	89.2
	390	26.8	4.6	2.8	44.7	15.5	4.5	69.3	93.8
Molasses	180	27.6	5.7	2.2	50.3	8.9	2.5	67.4	81.3
	280	28.3	4.3	4.4	46.7	11.5	3.2	65.7	83.6
	300	29.5	3.8	2.4	45.6	13.5	3.9	66.8	88.1

* γ-Linolenic acid (GLA), UFA : Total unsaturated fatty acid, DU : Degree of unsaturation (Double bonds / 100 molecules)

Summary

The influence of cultural conditions, especially glucose concentration, C/N ratio, and high concentration of molasses on cell growth and lipid accumulation of *Mortierella isabellina* IFO 7884 was investigated. The fungus was possible to grow at higher glucose concentration than 200 g/L glucose medium without deceleration. The high concentration of carbon source also accelerated lipid accumulation. The lipid content in the culture at high concentration of carbon source exceeded that at lower concentration of carbon source at the same C/N ratio. The maximum dry cell weight and total lipid production of 156.4 and 83.1 g/L, respectively, were obtained in the culture at 390 g/L of glucose concentration (C/N ratio = 20). The highest lipid productivity, 0.69 g/L/h, was obtained in the culture at lower glucose concentration, 270 g/L of glucose concentration. At the growth phase when lipid content was high, hyphal stretching was not observed and the fungal shape became yeast-like. This shape was considered to be beneficial to an increase in a fungal cell growth without increase in viscosity and without decrease in oxygen transport in culture broth.

CHAPTER III
Production of γ-Linolenic acid

Section 1 Production of γ-linolenic acid by genus *Mortierella*

Recently, research on microbiological production of γ-linolenic acid (GLA) and other polyunsaturated fatty acids (PUFAs), difficult to obtain from plant or animal oils, has been actively performed. However, all these researches have not been extended to practical production, because of their low PUFA content as well as low cell growth and low lipid productivity. As a method to raise cell mass concentration, fed-batch culture, in which carbon source was fed continuously during the culture, was also used by oleaginous yeast[61]. Repeated batch culture is also a kind of fed-batch culture, a portion of culture broth was taken out and the same amount of medium was replaced intermittently. In the previous chapter, the author made the cultural conditions of *Mortierella isabellina* clear and developed the possibility of single cell oil production[60] using the fungus of *Mortierella*[Chapter II, section 4].

This section deals with the investigation of influence of the cultural conditions at extremely high concentration of carbon source on GLA production by genus *Mortierella.* The cultivation mode of repeated batch culture was also investigated to increase in productivity of the lipid including GLA.

Materials and Methods

Microorganisms and cultural conditions. Genus *Mortierella* 6 species (9 strains), *M. isabellina* IFO 7824, 8183, 8308, and 8309, *M. vinacea* IFO 6738, *M. nana* IFO 8794, *M. ramanniana* IFO 8287, and *M. ramanniana* var. *angulispora* IFO 6744 and 8187 were obtained from the Culture Collection of the Institute of Fermentation, Osaka.

Basically, the culture medium contained the followings; 200 g glucose, 4.0 g urea, 2.25 g $(NH_4)_2SO_4$, 5.0g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.3 g NaCl, 0.6 g yeast extract, 0.6 g malt extract, 0.3 g pepton, 30 mg $FeSO_4 \cdot 7H_2O$, 30 mg $CaCl_2 \cdot 2H_2O$, 0.6 mg $CuSO_4 \cdot 5H_2O$, 3.0 mg $ZnSO_4 \cdot 7H_2O$, 3.0 mg $MnSO_4 \cdot 4H_2O$ and 1000 mL of distilled water. The C/N ratio of the medium was 34, and the various value of C/N ratio of 17 to 43 was adjusted by the amount of urea added. In case of the cultures changing glucose concentration, concentration of the other components were changed to have the same ratio to that of glucose. The nitrogen sources were sterilized separately with other components.

A 30 L stirred tank fermenter (Marubishi Rikasouchi, model MSJ-U2) system was employed in batch culture. The operation conditions were; 20 L working volume, 400-500 rpm agitation speed and 1.0 vvm air flow rate. The cultural pH was controlled at 4.0 with 2N NaOH. Culture was stopped when glucose concentration became zero. In cultural mode of repeated batch culture, double stage culture system (Kanto Rikaki Seisakusyo, model KRS-10-20) as shown in **Figure 1** was used.

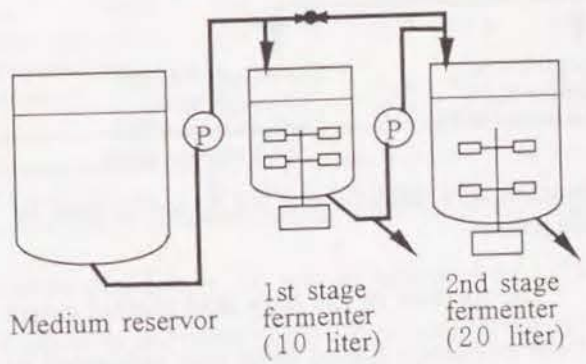


Figure 1. Double stage repeated batch culture system.

Analytical method. Analytical methods were the same as described previously in the section 1, Chapter I. The cell growth was determined by dry cell weight. Lipid was extracted from the mycelium, and amount of total lipid (TL, g/L-medium) was measured by weight. Fatty acid composition of TL was analyzed as methyl esters with gas chromatograph. Glucose concentration in culture broth was analyzed using a glucose analyzer (Yellow spring Instrument Co. Ltd., Model 27).

Content of GLA in the lipid was used as the value as GLA content in the fatty acid composition. A yield of GLA of the medium was used as the value of multiplied with the GLA content in TL and TL production of the medium. A productivity of GLA was evaluated by yield of GLA/culturing time.

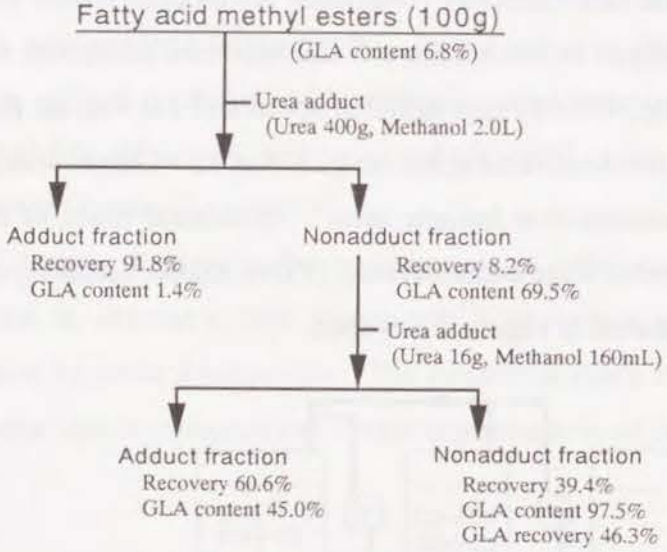


Figure 2. Concentration of γ -linolenic acid (GLA) methyl ester by the urea fractionation process.

Isolation of GLA. The mixture of the fatty acid methyl esters obtained by the esterification from extracted fungal lipid was subjected to the urea fractionation process[62]. The condition of re-crystallization of urea broth

is shown in **Figure 2** with results on recovery and GLA content of each fractions.

Results and Discussion

Comparison of GLA productivity among genus Mortierella. γ -Linolenic acid production at high glucose concentration was examined using 9 strains of the genus *Mortierella* (**Table 1**). The table contains the results obtained with *M. isabellina* IFO 7884 described previously in the section 4, Chapter II. As shown in **Table 1**, the cell growth of these strains were very high even at higher concentration of glucose more than 100 g/L, yielding from 40 to 156 g/L of dry cell weight and from 13 to 83 g/L of TL. A lipid contents were also very high (37 to 58%) for all the strain in **Table 1**.

Table 1. Production of lipids containing γ - linolenic acid (GLA) by genus *Mortierella*

Strain	IFO No.	Glu. (g/L)	Time (h)	DC (g/L)	TL (g/L)	TL/DC (%)	GLA in TL (%)	Y_{GLA} (g/L)
M. isabellina	7884*	390	168	156.4	83.1	53.1	4.5	3.7
〃	7884*	270	72	103.5	49.4	47.7	2.4	1.2
〃	7824	200	72	76.3	34.9	45.8	6.6	2.3
〃	8183	165	72	68.4	24.1	34.7	4.2	1.0
〃	8308	200	72	73.2	35.4	48.3	3.5	1.2
〃	8309	140	57	43.2	15.9	36.7	10.0	1.6
M. vinacea	6738	200	96	79.7	29.6	37.1	7.8	2.3
M. nana	8794	200	96	72.5	33.1	45.6	7.5	2.5
M. ramanniana	8287	200	96	69.8	27.6	39.5	5.8	1.6
M. ramanniana var. *angulispora*	6744	100	56	40.4	12.8	31.8	11.2	1.4
〃	8187	200	84	77.6	31.2	40.2	6.8	2.1

Glu : Glucose concentration as a carbon source Time : Incubation time
DC : Dry cell weight TL : Total lipid production Y_{GLA}:GLA yield
* The cell growth data were showed in the section4, Chapter II.

The content of GLA in TL was as high as 4 to 11% and was comparable with the content in the plant seeds of evening primrose. Further, the GLA yield on the culture medium was 1.2 to 3.7 g/L, and five strains of genus *Mortierella*, IFO 7884, IFO 7824, IFO 6738, IFO 8287, and IFO 8187,

attained GLA yield exceeding 2 g/L. Among these strains, *M. ramanniana* var. *angulispora* IFO 8187 had relatively high lipid productivity and high amount of GLA. Subsequent experiment of cultural conditions, therefor, was performed by the strain of *M. ramanniana* var. *angulispora* IFO 8187.

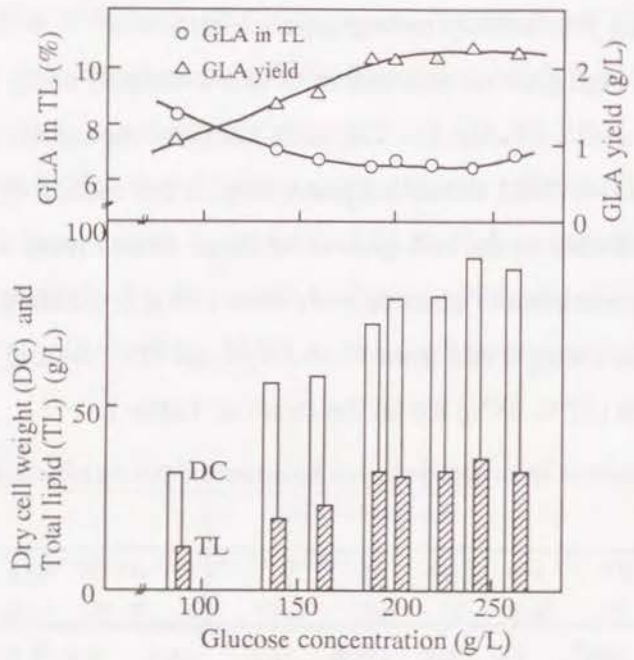


Figure 3. Effect of initial glucose concentration on cell growth, total lipid and GLA production.

Effect of cultural conditions on lipid and GLA production. The cultivation factors affecting the lipid and GLA production from glucose by the strain of *M. ramanniana* var. *angulispora* IFO 8187 were examined in detail. **Figure 3** shows the influence of glucose concentration on cell growth, lipid production and GLA yield. Both cell growth and lipid production were increased with the increase in glucose concentration up to 200 g/L. Increase in lipid production was not, however, observed at glucose concentration more than 200 g/L. The content of GLA in the TL was over 8%, at 90 g/L of glucose concentration and decreased, then, to 6.4% with the increase in glucose concentration up to 190 g/L. The GLA

content were almost constant at 6.5% with medium of glucose concentration more than 200 g/L. GLA yield showed a plateau in the culture at glucose concentration more than 190 g/L.

Effect of C/N ratio and incubation temperature on cell growth, lipid production and GLA yield were examined using medium at 200 g/L of glucose concentration (**Figure 4**). Culture condition at 34 of C/N ratio and 30℃ of incubation temperature gave the largest lipid production, but the content of GLA was the lowest instead. However, GLA yield became the highest value owing to the higher lipid production.

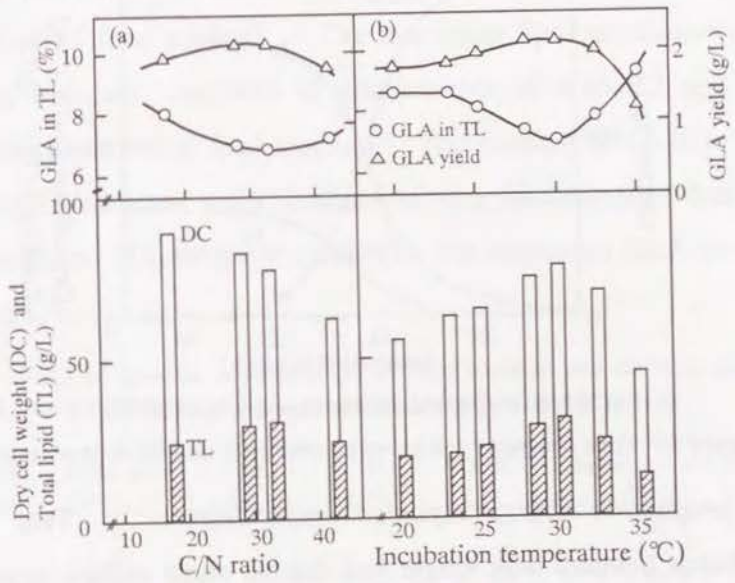
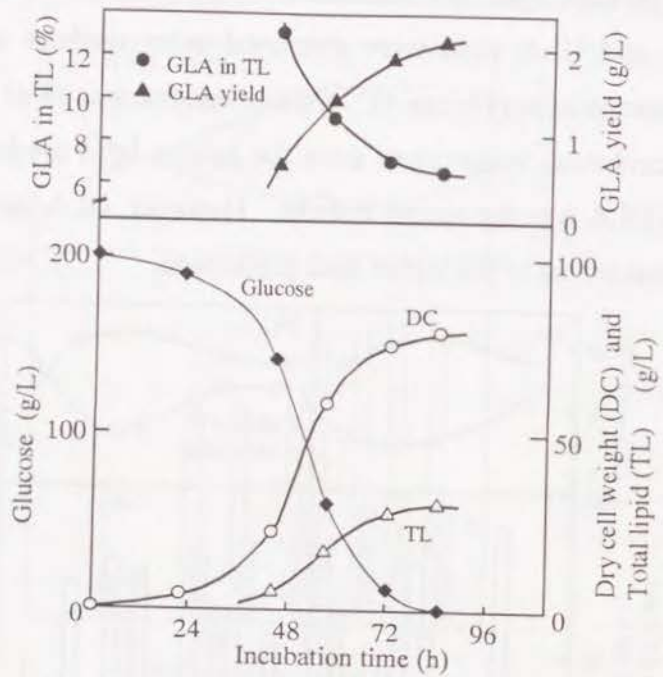


Figure 4. Effect of C/N ratio(a) and incubation temperature(b) on cell growth, total lipid and GLA production.

Time course of the dry cell weight, lipid production, GLA yield and glucose consumption of the culture under the above conditions are shown in **Figure 5**. As shown in the figure, glucose was consumed accordingly as the dry cell weight increased. Total lipid started to accumulate almost at 40 h incubation time, later than increase of cell growth. The content of GLA in TL was over 13%, at 44 h when the lipid production was still low,

then decreased to 7% according with the accumulation of lipid. However, yield of GLA became the highest valueof 2.2 g/L at 84 h owing to the higher lipid production.



(C/N ratio : 34, Incubation temperature : 30℃)

Figure 5. Time course of cell growth, total lipid, and GLA production.

Lipid production with a repeated batch culture. Two types of repeated batch cultures with single and double stage culture system were examined to obtain higher lipid and GLA productivity by *M. ramanniana* var. *angulispora* IFO 8187. Culture condition of a higher GLA content in the lipid generally gave a lower lipid productivity, and eventually lower GLA yield. Culture to obtain higher GLA yield was, consequently, attainable in a culture of high lipid productivity rather than high GLA content. The culture shown in **Figure 5** was used as a basis for comparison with repeated batch cultures. The lipid and GLA productivities

of batch culture, reference operational mode, were 8.9 g/L/d and 25 mg/L/h, respectively.

In a single stage repeated batch culture, a portion of culture broth was taken and replaced with an equivalent volume of new medium once a day. A series of experiments were undertaken to optimize the dilution rate (D), defined as replaced volume/working volume per day (d^{-1}), and glucose concentration of the feed medium. **Table 2** shows the results of the culture comparing with a batch culture. The maximum cell mass productivity of 35.0 g/L/d was obtained at a dilution rate of 0.67 d^{-1} and 200 g/L glucose concentration of feed medium. The maximum lipid productivity of 11.7 g/L/d was, however, observed at dilution rate of 0.33 d^{-1} and 300 g/L glucose concentration of feed medium. The content of GLA in the fatty acids of the TL obtained were comparable with the content of that obtained by batch culture. Under these conditions the maximum GLA productivity was 35 mg/L/h.

Table 2. Effect of glucose concentration of feed medium and dilution ratio on cell growth and lipid productivity with the single stage repeated batch culture

Feed medium glucose (g/L)	Dilution ratio (d^{-1})	DC (g/L)	TL (g/L)	TL/DC (%)	Productivity (g/L/h) P_C	P_L
200	0.50	61.6	22.2	36.0	30.8	11.1
200	0.67	52.6	15.4	29.4	35.0	10.3
300	0.33	89.8	35.2	39.2	29.9	11.7
300	0.50	65.7	20.8	31.7	32.8	10.4
300	0.67	47.8	10.7	22.4	31.8	7.1
(Batch culture*)	–	77.6	31.2	40.2	22.2	8.9

DC : Dry cell weight in the culture broth at stationary phase in repeated batch culture, TL : Total lipid weight in the culture broth at stationary phase in repeated batch culture, P_C : Productivity of cell mass, P_L : Lipid productivity
* Incubation time : 3.5 d

A double stage culture system was set up as shown in **Figure 1**. The working volumes of the first and second stage fermenters were 6.0 and 14.0 L, respectively. The first tank was intended for cell growth and the second tank for lipid accumulation. The volume of the second tank was made lager

in order to have a longer residence time for lipid accumulation. A typical time course of this operation is shown in **Figure 6**.

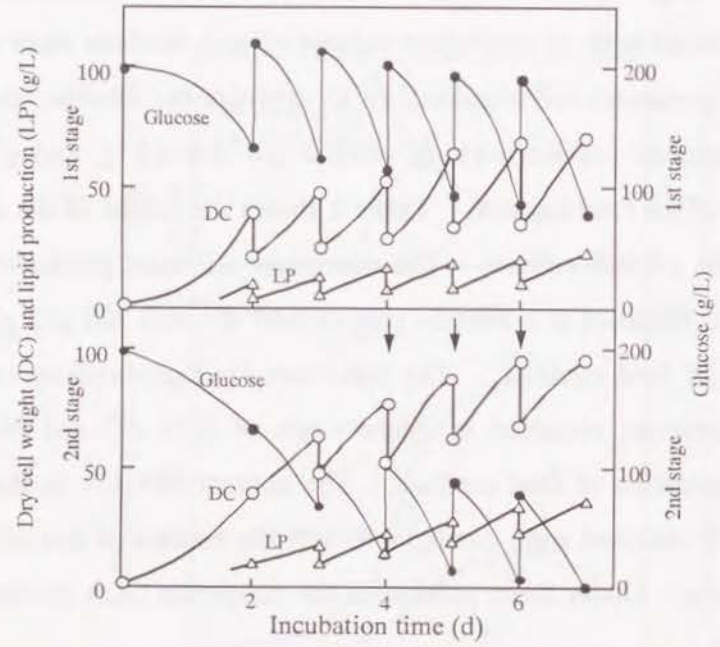


Figure 6. Time course of cell growth and lipid production in the double stage repeated batch culture. Working volume of the 1st tank:6L, the 2nd tank:14L. Each day, 3L of culture broth was removed from the 2 nd tank and the same amount of culture broth was transferred from the 1st tank and 3L of fresh medium (glucose 300g/L) was fed to the 1st tank. After 4 days incubation, volume of removed from the 2nd tank was increased to 6L and 3L of fresh medium was fed to the 2nd tank at arrows.

In a standard operation, 3 L of culture broth was removed from the second tank on day two of incubation time, and replaced by 3 L culture broth transferred from the first tank to the second tank and 3 L of fresh medium was fed to the first tank. On day four, 6 L of culture broth were removed from the second tank and transferred 3 L of culture broth from the first tank as before, plus 3 L of fresh medium was also fed to the second tank. The same operation was continued once a day until the termination of the experiment. The maximum lipid productivity with the method was 13.9 g/L/d, which was 20% and 50% larger than that of single stage repeated

batch culture and batch culture, respectively. γ -Linolenic acid productivity of repeated batch culture using double stage culture system was improved to 42 mg/L/h, 50% larger than that of batch culture.

Isolation of GLA. In order to obtain a purified GLA, urea fractionation process was carried out using mixture of fatty acid methyl esters obtained by the esterification of fungal lipid. Its procedure is shown in **Figure 2** with recovery and GLA content. From the mixture of 100g fatty acid methyl esters containing 6.8% of GLA, 15 g final product, GLA content of which was 97.5%, was obtained. Recovery of GLA was about 50%. Palmitic and oleic acid, whose amount was 30% and 45%, respectively, in the lipid, were completely separated out into urea adduct fraction. Relative low content of linoleic acid was convenient in purification like urea fractionation. Compared with evening primrose oil, which contains more than 70% of linoleic acid, much lower content of linoleic acid of the fungal lipid, 12%, was greatly beneficial to obtain purified GLA. This is superior point of the fungal oil obtained from *Mortierella* to evening primrose oil as a source of purified GLA, though GLA content of the fungal oil itself is similar to that of evening primrose oil.

Summary

Nine strains of genus *Mortierella* were cultivated batchwise at high glucose concentration for the purpose of improved GLA production. The cell growth of these strains were very rapid even at glucose concentration more than 100 g/L. As a result, five strains, including *M isabellina* IFO 7884 and *M. ramanniana* var. *angulispora* IFO 8187, attained GLA yield exceeding 2 g/L-medium. Among these strains, *M. ramanniana* var. *angulispora* IFO 8187 had relatively high lipid productivity and high amount of GLA. Using *M. ramanniana* var. *angulispora* IFO 8187, the effect of

various cultural conditions on GLA productivity were investigated. Culture condition at 34 of C/N ratio and 30 ℃ of incubation temperature gave the largest lipid production, but the content of GLA was the lowest value at this condition. However, yield of GLA became the highest value owing to the higher lipid production. Two types of repeated batch cultures with single and double stage culture system also were examined to obtain higher lipid and GLA productivity. The maximum lipid productivity of 13.9 g/L/h was obtained with the double stage repeated batch culture, which was 20% and 50% larger than that of single stage repeated batch culture and batch culture, respectively. γ -Linolenic acid productivity of the double stage repeated batch culture was improved to 42 mg/L/h. In order to obtain purified GLA, urea fractionation process was carried out using fatty acid methyl esters mixture of fungal lipid. The GLA content was finally concentrated up to 97% with the recovery of about 50%.

Section 2 Increase in the γ -linolenic acid content by solvent winterization of fungal oil extracted from *Mortierella* genus

The author searched fungi for purpose of production of γ -linolenic acid (GLA) by microorganisms, and found that *Mortierella ramanniana* var. *angulispora* IFO 8187 had relatively high lipid productivity and high amount of GLA[Chapter III, section 1]. Some trials of increasing GLA content for extracted fungal oil have been performed using adsorption to zeolites[63] or supercritical fluid chromatography[64,65]. Solvent winterization is one of the methods to separate oils and fats using the difference of melting point. Solvent winterization of sunflower seed oil[66,67] and effect of operational variables on the performance of the winterization[68] have been reported.

This section deals with increase in GLA content in fungal oil by solvent winterization, and also clarify the effect of solvent, oil concentration in solvents and temperature on various type of triacylglycerol (TG) and diacylglycerol (DG) and the separation efficiency for GLA (η_{GLA}) and TG types.

Materials and Methods

Fungal oil and solvents. The fungal oil extracted from *Mortierella ramanniana* var. *angulispora* IFO 8187, grown under the cultural conditions as described in the section 1, Chapter III, was used throughout the study. The solvents utilized for winterization were *n*-hexane, petroleum ether (boiling point 30 to 60 ℃), acetone, ethanol and chloroform, all of the analytical grade.

Winterization procedure. A fixed amount of the fungal oil was mixed with the appropriate amount of solvent in centrifugal tubes to make 5 %, 10

%, 20 % and 40 % oil in solvent by weight and cooled to 4℃ or -20℃ for 24 h or more until crystallization was complete. Separation of the crystallized fraction (CF) from the liquid fractions (LF) was carried out in Kubota Centrifuge KR/200A previously cooled to the same temperature. Centrifugation was done at 8,000 rpm (6,000 g) for 10 min followed by decantation of LF. The amounts of each resulting oil fraction was determined gravimetrically.

High performance liquid chromatography (HPLC). Separation and analysis of the TGs and DGs were carried out with HPLC using a Shimadzu Liquid Chromatograph LC-3A containing a 10 μL loop injector with a reverse phase column (Zorbax ODS, 250 mm x 6.2 mm I.D., DuPont Co.). The separated components were detected using a refractive index detector (Shimadzu, RID-3A). To determine the best separation of the TGs and DGs, combination of acetone/acetonitrile at a ratio of 4:1 (by vol.) as mobile phase was used. Samples of chloroform solution were injected and the HPLC was run isocratically at a flow rate of 1.0 mL/min. Triacylglycerol and DG standards, tripalmitin, triolein, trilinolein and 1,2-diolein, were obtained from Serdary Research Laboratories Inc., Ontario, Canada. Evening primrose oil, soybean oil and palm oil were used as TG mixture standards and their retention volume served as basis for the plots of log retention volume *vs.* theoretical carbon number (TCN)[69-72].

Analysis of lipid composition. The lipid composition and fatty acid composition were determined with TLC and GC by the methods as described previously in the section 1, Chapter I. The lipid distributions of the fungal oil and its separated fractions (CF, LF) were determined with TLC using the one-dimensional double development procedure. Quantitative analysis of the lipids separated on TLC were measured with a densitometer (Shimadzu CS - 910). Fatty acid composition of fungal oil

and its separated fractions (CF, LF) were also analyzed as fatty acid methyl esters (FAME) with gas chromatograph equipped with a flame ionization detector.

Results and Discussion

Lipid composition of the fungal oil. Analysis of the fungal oil from *Mortierella ramanniana* var. *angulispora* by TLC (**Table 1**) showed that the oil consisted mainly of TGs and 1,2-DGs.

Table 1. Lipid distributio of fungal oil of *M. ramanniana* var. *angulispora*

Lipid class	Composition (%)
Triacylglycerol	81.8
1,3-Diacylglycerol	3.4
1,2-Diacylglycerol	13.7
Free fatty acid	0.6
Sterol ester	0.4
Free sterol	0.1

Table 2. Fatty acid compositions of fungal oil of *M. ramanniana* var. *angulispora* analysed with GLC and HPLC

Fatty acid	GLC[a] (%)	HPLC[b] Mean ± SD (%)
16:0	31.9	32.7 ± 0.7
16:1	1.2	ND[d]
18:0	4.5	5.4 ± 0.2
18:1	46.0	47.3 ± 0.8
18:2	9.2	9.3 ± 0.7
18:3[c]	5.7	4.9 ± 0.4

a: GLC analysis of fatty acid composition.
b: The compositions obtained by calculation from HPLC analysis of TG and DG compositions of separated fraction after winterization process (number of samples 20).
c: γ-Linolenic acid. d: Not detected.

Analysis of the fatty acid composition revealed the presence of oleic and palmitic acids in major components as shown in the left column of **Table 2**. Content of GLA was 5.7%, based on total fatty acids.

Triacylglycerol and DG composition were analyzed by HPLC. The solvent mixture of acetone/acetonitrile at a ratio of 4:1 (by vol.) gave a good separation of TG mixture standards as well as fungal oil. The critical pairs with the same partition numbers (PN, in **Table 3**), such as OOO (54:3), POO (52:2) and POP (50:1), were clearly separated with the present method using the TG mixture standards (L: linoleic, O: oleic, S: stearic, P: palmitic. The designation LOP, POP *etc.*, does not imply the TG LOP but a mixture of all isomers: LOP, OLP, and OPL.). Identification of each TG was based on

comparison of retention volume and TCN[70] of both the TG of the mixture standards and the TG of the fungal oil. The theoretical carbon number was calculated from the following formula:

$$TCN = PN - (\Sigma_{Ui})$$

where Ui: a factor determined from several standards or TG mixture and was found to be: 0.65 for O, 0.85 for L, 0.2 for GLA and 0.0 for saturated acyl groups, Σ_{Ui}: the total Ui of individual fatty acids present in the TG.

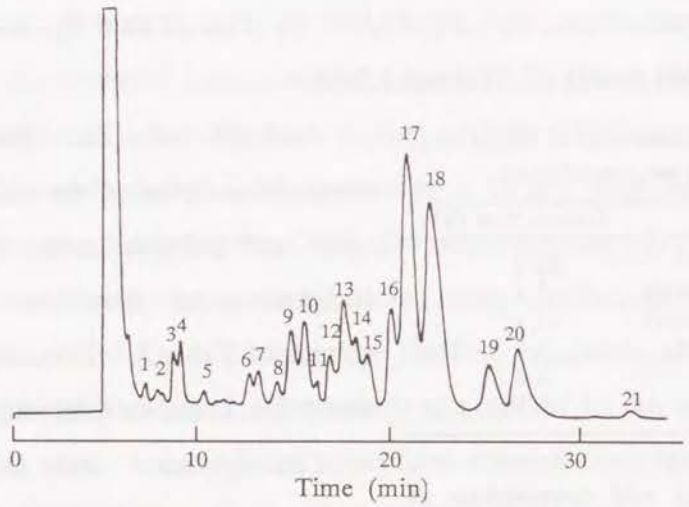


Figure 1. HPLC chromatogram of the fungal oil from *M. ramanniana* var. *angulispora*. Column, Zorbax ODS (250 mm × 6.4 mm I.D); and solvent system, acetone/acetonitrile (4:1 by vol.) at a flow rate of 1.0 mL/min. Peaks were identified as 4 diacylglycerols and 17 triacylglycerols; 1, GLAO; 2, OL; 3, OO; 4, PO; 5, GLALL; 6,GLALP; 7, LLO; 8, GLAOO; 9, GLALS; 10, GLAOP; 11, LOO; 12, LLS; 13, LOP; 14, LPP; 15, GLASP; 16, OOO; 17, POO;18, POP; 19, SOO; 20, SOP; and 21, SOS.

The peaks of chromatogram of fungal oil shown in **Figure 1** were identified as follows. Coincidence of retention volume of major peaks such as peak 13 and peaks 16 to 20 between TG mixture standards and fungal oil gave identification of these peaks, from which TCN relation was obtained (**Figure 2A**, **Table 3**). Other peaks from peak 5 to peak 21 were, then, identified using the TCN relation. Peak 5 to peak 21 were identified as 17 TGs, GLALL, GLALP, LLO, GLAOO, GLALS, GLAOP, LOO, LLS, LOP, LPP, GLASP, OOO, POO, POP, SOO, SOP, and SOS in the order of elution.

Table 3. Partition number (PN) and theoretical carbon number (TCN) of triacylglycerol and diacylglycerol

Glyceride	TC[a]:DB[b]	PN[c]	TCN[d]
Triacylglycerol			
GLALL[e]	54:7	40	38.1
GLALP	52:5	42	41.0
LLO	54:5	44	41.7
GLAOO	54:5	44	42.5
GLALS	54:5	44	43.0
GLAOP	52:4	44	43.2
LOO	54:4	46	43.9
LLS	54:4	46	44.3
LOP	52:3	46	44.5
LPP	50:2	46	45.2
GLASP	52:3	46	45.8
OOO	54:3	48	46.1
POO	52:2	48	46.7
POP	50:1	48	47.4
SOO	54:2	50	48.7
SOP	52:1	50	49.4
SOS	54:1	52	51.4
Diacylglycerol			
GLAO	36:4	28	27.0
LO	36:3	30	28.5
OO	36:2	32	30.7
PO	34:1	32	31.4

a: Total carbon number. b: Number of double bonds per molecule. c: PN = TC - 2 x DB. d: TCN = PN - Σ_{Ui} (Ui; O = 0.65, L = 0.85, GLA = 0.2). e: GLA, γ-linolenate; L, linoleate; O, oleate; S, stearate; P, palmitate The order of designation does not indicate the separation of positional isomers.

Peak 1 to peak 4 deviated from the line in **Figure 2A** and also peak 3 had the same retention volume as standard DG of 1,2-diolein. Retention volume and TCN of peaks of 1 to 4 also showed the same relationship as TG as shown in **Figure 2B**. The peaks of 1 to 4, therefore, were identified as DGs, that is, GLAO, OL, OO, and PO in the order of elution.

Glycerides composition of the fungal oil and its CF and LF are shown in **Table 4**. The predominant species were POO, POP, and LOP, whose contents were 24.4, 22.9, and 9.4%, respectively, of the total TG. In the right column of **Table 2**, the fatty acid composition from HPLC analysis is shown, indicating the comparison with that from GLC analysis of FAME.

The fatty acid composition from HPLC was obtained by calculation from TG and DG compositions of the fungal oil and of CF and LF in various conditions (number of samples 20) and expressed as mean ± SD. Analysis by HPLC gave a good agreement with that by GLC, which indicated validity of the identification of each TG and DG by HPLC analysis, though separation of each peak in **Figure 1** was not necessarily good because of many types of TGs.

Table 4. Comparative glyceride composition of the fungal oil and separated fractions with winterization process

Solvent	Temp. (℃)	C[a] (%)	F[b]	Triacylglycerol (%) GLALL[c]	GLALP	LLO	GLAOO	GLALS	GLAOP	LOO	LLS	Diacylglycerol (%) GLAO	OL	OO	PO
			Fungal oil	2.7	1.7	2.5	1.2	4.3	5.6	1.0	2.7	6.3	12.5	27.1	54.1
n-Hexane	-20	5	CF	-	-	-	-	0.6	-	-	-	-	-	23.8	76.2
			LF	2.5	1.7	2.7	1.2	5.2	7.0	1.2	3.6	7.0	15.8	31.6	45.6
		10	CF	-	-	-	-	-	0.6	-	-	-	-	18.2	81.8
			LF	2.4	2.1	3.2	1.6	5.6	7.5	1.3	4.1	8.8	14.0	31.6	45.6
		20	CF	-	-	-	-	0.5	0.9	-	0.2	-	25.8	16.1	58.1
			LF	2.5	2.2	3.1	1.2	6.0	8.8	1.4	4.4	8.3	15.0	30.0	46.7
		40	CF	-	0.9	0.8	-	2.3	3.1	-	1.0	-	-	-	98.0
			LF	2.2	2.5	3.6	1.7	5.7	8.0	1.6	4.3	7.4	14.8	27.8	50.0
	4	40	CF	-	0.7	1.1	0.5	2.5	3.1	-	0.3	-	-	32.0	68.0
			LF	1.6	1.9	2.9	1.4	5.1	7.1	1.4	3.8	8.6	13.8	29.3	48.3
Petroleum ethere	-20	5	CF	-	-	-	-	0.1	0.9	0.5	1.7	-	-	-	99.9
			LF	2.6	2.1	3.6	2.1	5.2	6.8	1.6	3.5	7.4	14.8	29.6	48.2
		10	CF	0.2	0.4	0.7	-	0.2	1.3	1.7	2.9	-	-	-	99.9
			LF	1.5	2.0	2.5	1.1	5.0	6.3	0.8	3.2	5.3	14.0	33.3	47.4
		20	CF	0.3	0.6	2.3	0.6	0.9	1.4	0.3	0.5	-	30.8	25.6	43.6
			LF	1.7	2.4	3.6	1.6	6.0	7.1	1.6	3.8	5.8	13.4	30.8	50.0
		40	CF	-	0.4	0.7	0.7	1.6	2.6	0.8	1.3	-	-	39.1	60.9
			LF	2.6	1.5	2.7	1.1	4.8	6.8	1.0	3.2	6.7	13.3	26.7	53.3
Acetone	-20	5	CF	-	-	-	-	0.9	1.7	0.7	0.3	-	-	36.4	63.6
			LF	1.7	3.1	4.2	2.1	6.9	8.9	1.2	4.4	6.8	12.3	28.8	52.1
		10	CF	-	0.4	0.7	-	1.3	2.0	0.9	1.0	-	-	38.5	61.5
			LF	2.1	2.9	3.8	1.5	7.3	9.2	0.8	5.0	5.9	12.9	30.6	50.6
		20	CF	-	0.5	0.9	0.4	2.8	3.9	1.2	1.6	-	-	38.5	61.5
			LF	2.3	3.7	4.8	1.8	8.2	9.6	0.7	5.1	8.7	7.8	30.1	53.4
		40	CF	-	0.9	1.9	0.5	3.8	4.8	0.6	1.3	-	16.7	30.0	53.3
			LF	2.7	4.1	5.4	2.6	8.2	10.3	-	7.0	7.3	15.4	32.0	45.3
	4	5	CF	-	0.6	0.9	0.5	2.1	2.7	0.7	1.4	-	12.0	32.0	56.0
			LF	2.4	2.8	3.4	1.3	6.3	8.0	1.2	3.7	6.0	14.9	31.1	47.8
		10	CF	-	0.8	1.2	0.8	2.4	2.9	1.0	1.4	-	17.4	30.4	52.2
			LF	2.5	2.5	3.6	1.4	6.1	8.2	1.5	4.2	8.5	14.1	29.6	47.9
		20	CF	-	0.7	1.0	0.6	2.5	3.2	0.8	1.8	-	16.0	32.0	52.0
			LF	2.4	2.2	3.2	1.5	6.5	8.8	1.3	4.6	7.3	15.8	29.3	47.6
		40	CF	0.4	1.0	1.4	0.8	3.3	5.0	1.2	2.4	-	13.9	30.6	55.5
			LF	2.8	2.4	3.3	1.4	6.0	7.7	1.2	4.0	9.7	17.1	29.3	43.9
Ethanol	4	5	CF	-	0.6	1.5	0.7	2.7	3.9	1.1	1.4	-	25.0	33.3	41.7
			LF	2.5	5.6	5.5	3.1	10.3	10.6	-	9.6	8.6	15.3	20.3	56.1
Chloroform	-20	40	CF	-	0.8	1.3	2.2	2.2	3.6	-	1.2	-	-	34.6	65.4
			LF	1.2	1.7	2.4	1.5	5.0	6.4	1.1	3.6	9.8	8.2	31.1	50.9

a:Concentration of oil in solvent(%w). b:The fraction separated with winterization (CF; crystallized fraction; LF, liquid fraction). c:GLA, γ -linolenate; L, linoleate; O, oleate; S, stearate; P, palmitate.

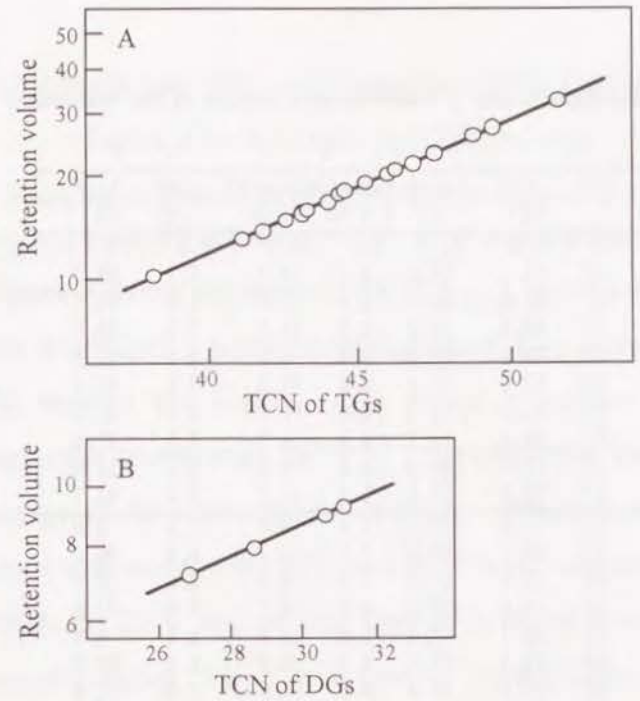


Figure 2. Theoritical carbon number (TCN) of triacylglycerols (TGs)(A) and diacylglycerols (DGs)(B) vs. ther retention volume of fungal oil.

Winterization of the fungal oil. **Table 5** summarizes the yields of fraction (Y_f), lipid composition, GLA content and yield of GLA (Y_{GLA}) of the CF and LF of the fungal oil after winterization.

Increase in oil concentration in solvents, as well as decrease in the winterization temperature, decreased Y_f in LF. Triacylglycerol was concentrated into CF but DG into LF. Ethanol provided the highest concentration of TG into CF (92.3% of lipid), in spite of high yield of CF (73.7%). The high concentration of TG seems to be brought about by high polarity of ethanol and very low TG solubility in ethanol.

γ -Linolenic acid contents of LF (6.0-10.5%), were higher than the original oil (5.7%), owing to comparatively high solubility of GLA. The fractions which had more than 10% GLA content in LF, however, showed both very low yield of LF (18.3% with acetone at -20℃ and 26.3% with ethanol at 4℃) and relatively low Y_{GLA} at less than 50%.

Table 5. Yields, lipid distribution and γ-linolenic acid content of the winterized fraction of the fungal oil

Solvent	Temp. (℃)	C[a] (%)	F[b]	Y_f[c] (%)	Lipid distribution(%) TG	1,3-DG	1,2-DG	Ln[d] (%)	Y_{GLA}[e] (%)
n-Heaxne	-20	5	CF	24.8	87.2	2.7	10.0	1.7	7.3
			LF	75.2	75.5	4.3	18.4	7.1	92.7
		10	CF	31.8	88.4	2.8	8.6	1.8	10.1
			LF	68.2	75.3	4.2	19.0	7.5	89.9
		20	CF	36.0	87.6	3.4	8.8	2.0	12.9
			LF	64.0	75.1	3.9	19.2	7.6	87.1
		40	CF	41.6	84.9	4.0	10.1	3.0	22.2
			LF	58.4	73.7	6.5	18.3	7.5	77.8
	4	40	CF	27.5	86.9	4.4	8.3	3.5	15.9
			LF	72.5	79.5	5.4	14.7	7.0	84.1
Petroleum ether	-20	5	CF	19.9	93.0	1.4	5.6	1.4	4.8
			LF	80.1	80.0	3.7	15.4	6.9	95.2
		10	CF	21.4	87.9	2.9	9.2	2.0	7.2
			LF	78.6	78.3	4.0	16.7	7.0	92.8
		20	CF	20.7	88.3	2.9	8.8	2.4	8.4
			LF	79.3	78.0	4.2	16.6	6.8	91.6
		40	CF	27.0	86.4	2.6	11.0	3.0	13.9
			LF	73.0	79.1	3.9	15.7	6.9	86.1
Acetone	-20	5	CF	44.4	92.4	2.3	5.0	2.7	21.0
			LF	55.6	72.1	4.4	21.9	8.1	79.0
		10	CF	49.7	89.1	2.0	8.4	2.7	24.3
			LF	50.3	73.3	5.2	19.6	8.3	75.7
		20	CF	63.8	90.9	0.8	8.0	3.6	39.6
			LF	36.2	70.7	5.9	20.7	9.7	60.4
		40	CF	81.7	87.6	2.1	10.0	4.7	67.6
			LF	18.3	62.5	6.9	29.5	10.1	32.4
	4	5	CF	46.4	92.5	1.1	6.0	3.7	29.9
			LF	53.6	73.5	5.7	18.8	7.5	70.1
		10	CF	46.8	90.5	2.3	6.9	3.5	29.1
			LF	53.2	76.9	4.2	17.4	7.5	70.9
		20	CF	56.8	88.6	2.3	8.7	4.2	40.8
			LF	43.2	73.1	4.9	19.8	8.0	59.2
		40	CF	67.3	84.5	3.5	11.5	4.8	55.6
			LF	32.7	74.2	4.6	19.7	7.9	44.4
Ethanol	4	5	CF	73.7	92.3	2.0	4.9	3.9	51.0
			LF	26.3	53.5	7.8	30.5	10.5	49.0
Chloroform	-20	40	CF	33.4	86.1	2.8	10.3	4.0	25.1
			LF	66.6	78.7	2.5	17.8	6.0	74.9

a: Concentration of oil in solvent (%w). b: The fraction separated with winterization (CF, crystallized fraction; LF, liquid fraction). c: Yield of each separated fraction. d: Content of γ-linolenic acid by GLC analysis. e: Yield of γ-linolenic acid.

In order to evaluate the effectiveness of separation including GLA yield, separation efficiency for the substance S into fraction LF [(η_S) defined as the following equation] is introduced:

$$\eta_S = (Y_{LF}x_l / 100x_f) - (Y_{LF}(1 - x_l / 100) / (100 - x_f))$$
$$= (x_f - x_c)(x_l - x_f) / x_f(1 - x_f / 100)(x_l - x_c)$$

where Y_{LF} = Yield of fraction LF (%); x_f = Content of S in fungal oil (%); x_l = Content of S in fraction LF (%); x_c = Content of S in fraction CF (%).

Figure 3 shows the dependence of η_{GLA} on oil concentration in solvent. Higher winterization temperature decreased η_{GLA} and oil concentration of 40 % showed the least η_{GLA} except petroleum ether. Effect of winterization temperature on η_{GLA} revealed that lower temperature was advantageous for concentration of GLA. The highest η_{GLA} (0.27) was obtained with acetone at -20℃ and at 10% oil concentration in the solvent. Acetone, at -20℃ and at less than 20% of oil concentrations, indicated comparably higher η_{GLA}. In case of lower oil concentration (5 - 20%), generally, η_{GLA} showed higher in the following order: acetone (-20℃) > *n*-hexane (-20℃) > acetone (4℃) > petroleum ether (-20℃). Oil concentration at 5%, ethanol (4℃) showed high value of η_{GLA}, indicative of the effect of lower solubility of TG without GLA than TG with GLA.

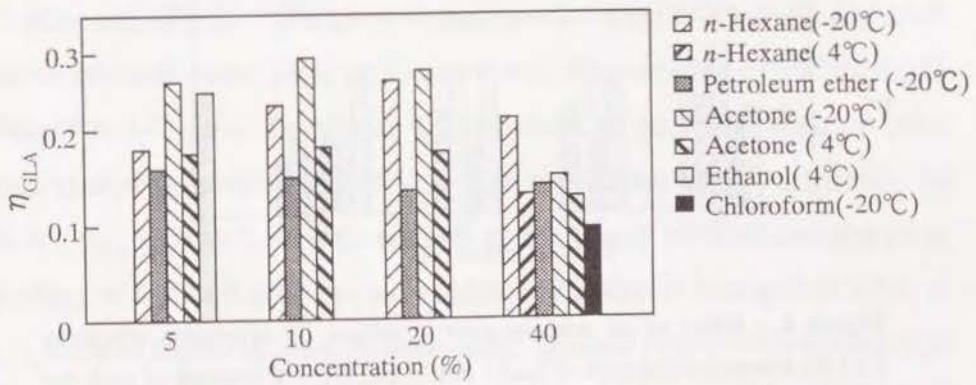


Figure 3. Effect of oil concentration in solvent on separation efficiency for γ-linolenic acid (η_{GLA})

Among the main TG mentioned above, disaturated TGs (Sa_2U; Sa, saturated fatty acid; and U, unsaturated fatty acid; POP, POS, SOS, *etc.*)

were concentrated into CF, whereas diunsaturated TGs (SaU_2, OOP, LOP, LLS, *etc.*) and triunsaturated TGs (U_3, GLALL, LLO, OOO, *etc.*) were concentrated into LF. These results indicated that the winterization was effective for the separation of Sa_2U and SaU_2. As for the concentration of GLA, most TGs containing GLA were separated into LF.

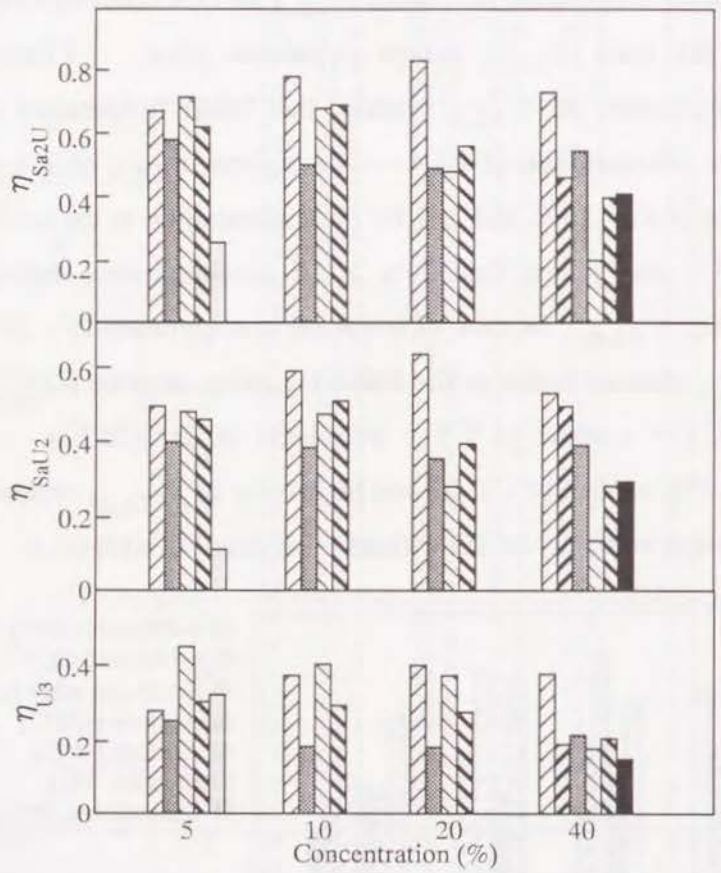


Figure 4. Effect of oil concentration in solvent on separation efficiency (η) for triacylglycerol type of Sa_2U, SaU_2, and U_3. Condition of each bar was same as Figure 3.

Figure 4 shows the separation efficiencies for η_{Sa2U} into CF, η_{SaU2} and η_{U3} into LF, respectively, *vs.* oil concentration in solvents. Separation efficiency for Sa_2U showed high value (0.7-0.8) with *n*-hexane throughout the oil concentration examined, and η_{Sa2U} showed relatively high values (> 0.5) with all the solvents examined in the range of oil concentration less than 20%. Disaturated TGs, Sa_2U and especially Sa_2O such as POP, POS and SOS are the main components of cocoa butter which has a melting point near at 35℃ [73]. The present method, therefore, revealed to be an excellent one to obtain a cocoa butter like fat into CF. Higher η_{SaU2} was obtained with *n*-hexane than with any other solvent. In case of U_3, however, low oil concentration in acetone (-20℃) and ethanol showed high η_{U3} which was correlated to the high η_{GLA} with the solvent.

Summary

The fungal oil extracted from *M. ramanniana* var. *angulispora* IFO 8187, GLA content 5.7%, was used in order to raise the content of GLA by solvent winterization. Effects of winterization conditions (solvent, oil concentration in the solvent, and temperature) and varieties of glyceride compositions were discussed. The fungal oil was separated into 4 DGs and 17 TGs with HPLC. The predominant species were POO, POP, and LOP, whose contents were 24.4, 22.9, and 9.4%, respectively, of the total TG. Ethanol at 4℃ gave the highest GLA content of 10.5% in spite of lower yield than with acetone at -20℃. The highest separation efficiency for GLA (η_{GLA}) was 0.27 with acetone at -20℃ and 10% oil concentration, resulting in 8.3% GLA. In case of lower oil concentration at 5 to 20%, η_{GLA} showed higher in the following order: acetone (-20℃) > *n*-hexane (-20℃) > acetone (4℃) > petroleum ether (-20℃). The winterization process also proved to be effective for the separation of TG type, Sa_2U (Sa: saturated fatty acid, U: unsaturated fatty acid) into the crystallized fraction and SaU_2 into the liquid fraction. Acetone at -20℃ showed higher separation efficiency for triunsaturated TG than the other solvents.

Section 3 Production of Lipid containing γ -linolenic acid by continuous culture of *Mortierella ramanniana*

A higher productivity of cell mass and primary metabolite are generally expected in continuous culture than batch culture. In case of microbial lipid production, however, continuous culture has not been estimated better than batch culture, because lipid accumulative phase is situated at later logarithmic growth phase or at stationary phase. Continuous culture of a lipid accumulative yeast, genus *Rhodotorula*, in fact, resulted in a low lipid productivity (P_L) owing to lower lipid content even if higher dilution rate (D) close to its maximum growth rate gave higher productivity of the cell (P_C)[51,52,74]. Culture conditions of nitrogen limitation as well as lower D are to be chosen for higher P_L in continuous culture. These conditions, however, are unfavorable for growth, which resulted in lower P_L than that of batch culture. In case of γ -linolenic acid (GLA) production using genus *Mucor*, Hansson *et al.*[75] used a medium at high concentration of nitrogen source and resulted in a low P_L owing to lower growth as well as lower lipid content. The author has obtained a high GLA productivity as well as high cell mass in batch culture after investigation of the effect of culture conditions using *Mortierella ramanniana* as described previously in the section 1 of this Chapter.

This section deals with the investigation of the effect of continuous operation on lipid and GLA productivity and compares with the data of batch operation.

Materials and Methods

Microorganisms and cultural conditions. The strain of *Mortierella ramanniana* var. *angulispora* IFO 8187 was used in this study. Basically,

the liquid culture medium contained the followings; 100 g glucose, 5.0g $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.15g NaCl, 0.3 g malt extract, 0.3 g yeast extract, 0.15 g pepton, 15 mg $FeSO_4 \cdot 7H_2O$, 1.8 mg $CaCl_2 \cdot 2H_2O$, 1.5 mg $CuSO_4 \cdot 5H_2O$, 1.5 mg $ZnSO_4 \cdot H_2O$, and 1.5 mg $MnCl_2 \cdot 4H_2O$ in 1000 mL of distilled water. In case of the cultures changing glucose concentration, concentration of the other components were also changed to have the same ratio to that of glucose. In the culture changing concentrations of malt extract and yeast extract or C/N ratio, however, only concentration of these extracts or $(NH_4)_2SO_4$ was changed.

Continuous culture were performed using 1.25 L continuous stirred tank reactor (New Brunswick Science Co. Ltd., Model C-32). New medium was fed at a constant flow rate and the volume of culture medium was kept constant with over flow exit. Dilution rates ranged from 0.05 to 0.20 h^{-1}. Value of pH was kept at 4.0 using 1 N NaOH, culture temperature at 30℃, impeller speed at 600 rpm, and aeration rate at 1.0 vvm.

Analytical methods. Culture broth of 25 mL were taken out to analysis of cell mass and lipid accumulation. The cell mass was determined by dry cell weight. Glucose concentration in culture broth was analyzed using a glucose analyzer (Yellow Spring Instrument Co. Ltd., Model 27). Several samples at 3 to 4 h's intervals were taken out and measured to make it certain whether the culture reached to steady-state conditions at given culture conditions. Samples at steady-state condition, then, were subjected to lipid extraction and lipid analysis by the methods as described previously in the section 1, Chapter I. Lipid was extracted from the cell, and the total lipid production was measured by weight. The extracted lipids were fractionated into neutral lipids (NL) and polar lipids (PL) by silicic acid chromatography. Lipid distributions of NL and PL were performed by TLC,

and fatty acid compositions of each lipids were performed by GC after methanolysis.

Productivitys of P_C and P_L were calculated from cell mass (g/L) and total lipid production (g/L), respectively, multiplied by D. Product yield, or lipid yield, was the percentage of (lipid produced)/(glucose consumed).

Result and Discussion

Effect of dilution rate on cell mass and lipid productivity. Continuous culture with inlet glucose concentration (G_I) at 60 g/L and various D from 0.05 to 0.20 h^{-1} were conducted (**Figure 1**).

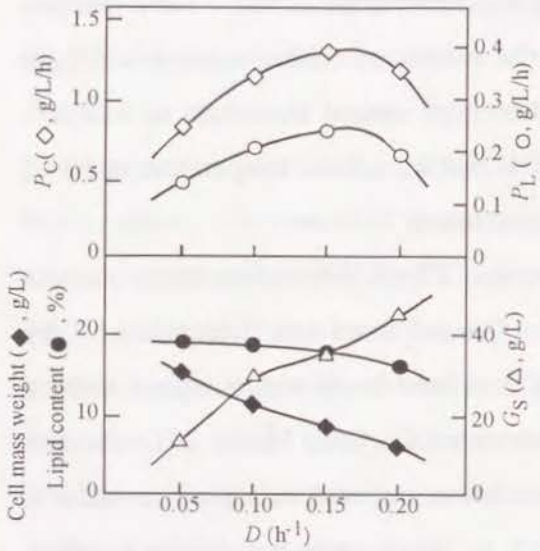


Figure 1. Effect of dilution rate (D) on cell mass productivity (P_C), lipid productivity (P_L), lipid content, and glucose concentration at steady-state (G_S).

(Inlet glucose concentration, G_I = 60 g/L)

Glucose concentration at steady-state (G_S) increased and cell mass decreased according to D. Lipid content, however, indicated a comparatively constant value. At 0.15 of D, both P_C and P_L resulted in the maximum values . Though wash-out point was not directly checked, 0.20 of D was sufficiently estimated to be very near to the wash out point owing

to low cell mass and high G_S at D = 0.20 h^{-1}. From the point of both P_C and P_L, 0.15 h^{-1} of D was chosen as the optimum D at 60 g of G_I.

Effect of inlet glucose concentration and dilution rate. In order to find further the optimum conditions for P_C and P_L, continuous cultures at various inlet glucose concentrations from 60 to 210 g/L and at D from 0.05 to 0.20 h^{-1} were examined. **Figure 2** shows the result. The highest P_L was obtained at 0.10 of D and at 180 g/L of G_I. Dilution rates for higher P_L were 0.10 for more than 150 g/L of G_I, but were 0.15 for less than 120 g/L of G_I. It is natural for the fungus to require a longer residence time, or smaller D, to utilize higher concentration of glucose. This is also attributed to be an inhibition by high concentration of glucose. Effect of this inhibition was indicated by abrupt drop of P_L at higher G_I.

Curves of G_S had a turning point at about 100 g/L (shown as dotted line in **Figure 2**), over which point increment of G_S was increased, or growth efficiency was decreased. Though the meaning of this turning point at 90 g/L was not clear, some factors other than glucose concentration was thought to form growth-limiting step. Without growth-limiting nutrient, further, P_C, or P_L, should have been fairly proportional to D, or growth rate. But that is not the case with the culture as shown in **Figure 2**, suggestive of growth-limiting by some nutrient other than glucose again.

Concentration of yeast and malt extracts. In order to find the growth limiting factor, cultures at elevated concentration of yeast extract and malt extract at the constant G_I (100 g/L) were performed, whose result is shown in **Figure 3**. Increase in yeast and malt extracts resulted in increase in P_L, indicating of some limiting factor in these extracts. Though P_L increased according to increase in those extracts' concentrations, increment of P_L increase from 0.6 g/L to 0.9 g/L was smaller than that from 0.3 g/L to 0.6 g/L. The values of G_S at 0.6 and at 0.9 g/L, further, resulted in the very

similar value, indicating no more increase in lipid yield between 0.6 and 0.9 g/L of extracts concentration. Subsequent experiment, therefore, was performed at 0.6 g/L of the extracts concentration.

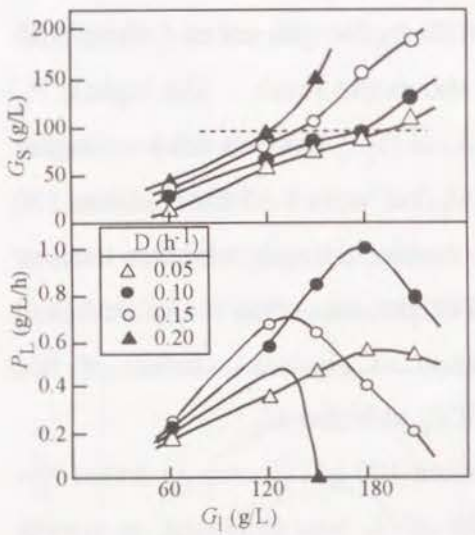


Figure 2. Change of lipid productivity (P_L) and glucose concentration at steady state (G_S) at various glucose concentration (G_I) and dilution rate (D).

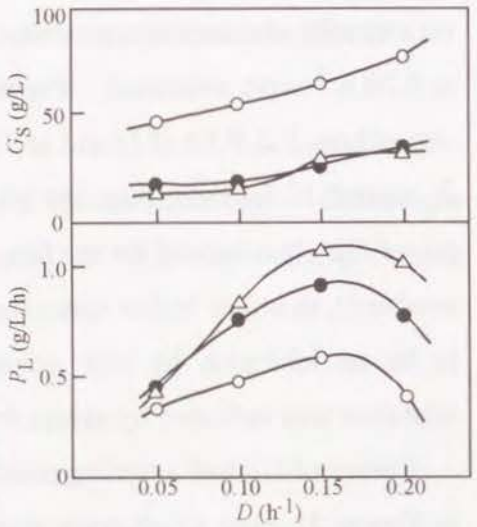


Figure 3. Effect of extracts concentration on lipid productivity (P_L) and glucose concentration at steady-state (G_S) at various dilution rate (D). Inlet glucose concentration was 100 g/L. Yeast extract and malt extract contents were 0.3 g/L (○), 0.6 g/L (●) and 0.9 g/L (△).

Effect of C/N ratio. Effect of C/N ratio was checked by changing $(NH_4)_2SO_4$ concentration at 100 g/L of G_I, 0.6 g/L of both extract concentration and 0.15 of D, whose result is shown in **Figure 4**. The values of P_C started to decrease at the range of C/N ratio higher than 23.4, showing limiting of nitrogen source on cell growth. Lipid content increased from about 20% to 30% according to increase in C/N ratio as shown in many case. The maximum P_L of 1.08 g/L/h was obtained with the culture

at 23.4 of C/N ratio. After optimization of nitrogen and extracts content, higher P_L was obtained at 100 g/L of G_I than that at 180 g/L (about 0.99 as shown in **Figure 2**). Under this condition, G_S was 20 g/L, which also gave higher lipid yield of 10.0%. Comparing with batch culture, described in section 1 of this Chapter, in which the maximum P_L was obtained at 34 of C/N ratio, the maximum P_L was obtained at lower C/N ratio (23.4) in continuous culture.

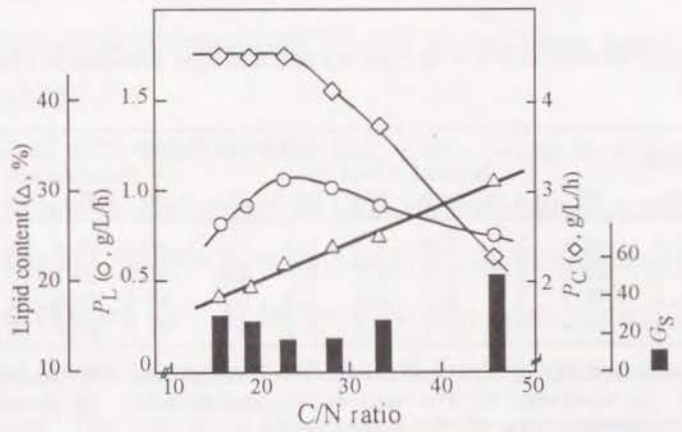


Figure 4. Effect of C/N ratio on cell mass productivity (P_C), lipid productivity (P_L), lipid content, and glucose concentration at steady-state (G_S). Inlet glucose concentration was 100 g/L. Yeast extract and malt extract concentration was 0.6 g/L. Dilution rate was 0.15 h^{-1}.

Culture effect on lipid content and fatty acid composition. Effect of D on lipid distribution and fatty acid composition were also examined for the culture at 100 g/L of G_I and 0.6 g/L of extracts content. **Table 1** shows the effect of D on lipid content and lipid distribution. Increase in D resulted in decrease in lipid content. Main contribution of the decrease of lipid content came from the decrease in NL content, but not from PL content. Growth in continuous cultures at lower D and higher D, respectively, can correspond to early and late growth phase of batch culture. In batch culture,

lipid accumulation occurred mainly at and after logarithmic growth phase. This result, therefore, indicated the coincidental tendency of the continuous culture with batch culture. As for each lipid distribution of NL and PL, main cause of the decrease existed in triacylglycerol, the most abundant component. Free fatty acid and 1,2-diacylglycerol, on the other hand, increased greatly, indicating inhibition of triacylglycerol formation at higher *D*. Content of the main polar lipid distribution, phosphatidylcholine and phosphatidylethanolamine, did not substantially changed.

Table 1. Effect of dilution rate (*D*) on lipid content and lipid distribution of neutral and polar lipid

D	NL/DC	PL/DC	Lipid distribution										
			Neutral lipid (%)						Polar lipid (%)				
(h^{-1})	(%)	(%)	TG	1,3-DG	1,2-DG	FFA	SE	FS	PC	PE	PS	PI
0.05	28.3	2.9	84.4	2.6	1.8	1.7	1.8	7.7	49.3	40.4	4.0	0.6
0.10	24.9	3.3	80.9	0.7	6.2	4.7	1.6	5.6	51.2	38.7	2.1	0.9
0.15	20.5	3.3	76.3	1.7	8.2	4.0	1.3	6.6	49.7	39.3	4.9	0.8
0.20	17.6	4.0	76.4	1.2	10.1	5.0	1.2	6.1	47.6	41.6	6.0	0.7

NL/DC: Neutral lipid content PL/DC: Polar lipid content TG: Triacylglycerol DG: Diacylglycerol FFA: Free fatty acid SE: Sterol ester FS: Free sterol PC: Phosphatidylcholine PE: phosphatidylethanolamine PS: Phosphatidylserine PI: Phosphatidylinositol

Table 2. Effect of dilution rate (*D*) on neutral lipid and polar lipid fatty acid composition

D	Fatty acid composition											
	Neutral lipid (%)						Polar lipid (%)					
(h^{-1})	16:0	16:1	18:0	18:1	18:2	18:3*	16:0	16:1	18:0	18:1	18:2	18:3*
0.05	30.4	2.6	5.1	44.6	9.4	6.6	13.7	2.0	1.2	23.5	34.1	24.2
0.10	28.1	1.9	4.3	43.5	13.6	6.9	13.3	1.8	2.7	23.2	33.3	25.0
0.15	30.1	1.1	4.8	44.6	10.6	7.6	13.7	1.4	0.3	22.3	34.9	26.1
0.20	29.2	2.1	3.5	45.5	9.9	7.4	13.7	1.8	0.9	33.1	26.8	21.7

* γ-Linolenic acid

Table 2 shows the fatty acid composition of each lipid fraction. Major fatty acids in NL were palmitic and oleic acid, the content of which were affected little by the change of *D*. Higher content of linoleic acid was obtained at 0.1 h^{-1} of *D*, and increase in *D* showed the tendency of decrease from 13.6 % to 9.9 %. Content of GLA increased instead according to

increase in *D*. In PL, there seems to be no substantial change of fatty acid composition except a little increase in GLA according to increase in *D*. At 0.20 h^{-1} of *D*, there occurred 10% higher content of oleic acid as well as lower linoleic acid and GLA, presumably related to be very near to wash out point. Kendrick and Ratledge[76] reported main factor for changing polyunsaturated fatty acid composition was temperature in continuous culture of oleaginous fungus (*Entomorphthora exitalis*). Increase in linoleic acid content according to increase in *D* was reported with a thermotolerant yeast (*Hansenula polymorpha*)[77] and a oleaginous yeast (*Trichosporon pullulans*)[78].

Effect of C/N ratio on fatty acid composition is shown in **Table 3**. Content of linoleic acid as well as GLA showed a little increase according to the increase in C/N ratio from 15.1 to 27.8. In this region of C/N ratio, G_S decreased (**Figure 4**). At higher C/N ratio when growth was repressed, content of linoleic acid and GLA in total lipid decreased due to increase in NL content. The maximum GLA productivity was calculated to more than 100 mg/L/h in the condition at 23.4 of C/N ratio which gave the highest P_L.

Table 3. Effect of C/N ratio on total lipid fatty acid composition

C/N	Fatty acid composition (%)						UFA
ratio	16:0	16:1	18:0	18:1	18:2	18:3*	(%)
15.1	28.3	3.2	2.6	42.1	12.7	9.0	68.1
18.8	27.1	2.8	3.2	42.7	13.1	9.6	68.6
23.4	24.7	1.5	2.7	44.9	13.7	9.7	70.7
27.8	26.9	3.2	2.1	42.9	13.9	9.8	70.6
33.8	25.7	1.2	3.8	47.7	12.5	8.4	70.1
46.0	25.8	1.9	4.1	47.8	12.6	6.8	69.1

Dilution rate = 0.15 h^{-1} * γ-Linolenic acid UFA: Total unsaturated fatty acid

Characteristics of the fungus' growth and lipid accumulation. In most cases of oleaginous yeasts, lower *D* was chosen for lipid production, in spite of lower growth rate, owing to higher lipid content. In the fungus of the present work, variation of lipid content has appeared to be relatively small.

Graphic design for continuous culture from the growth curve of batch culture at 90 g/L of G_I and 34 of C/N ratio is shown in **Figure 5**, where growth rate (d*x*/d*t*) *vs*. dry cell weight (*x*) is depicted[79].

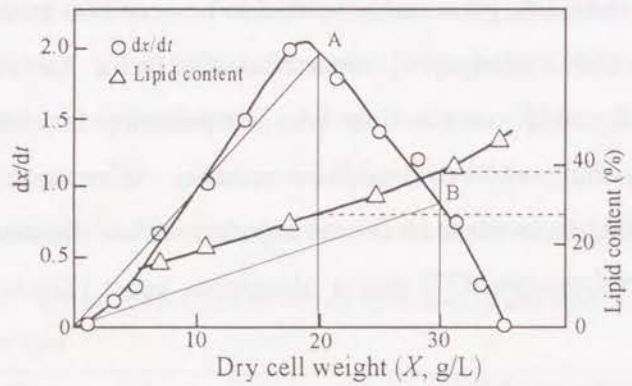


Figure 5. Graphic design for continuous culture from the growth curve of batch culture. Initial glucose concentration was 90 g/L. C/N ratio was 34.

Two points, A and B, correspond to 0.10 and 0.03 of D, respectively. At 0.10 of D (point A), dry cell weight is to be 20 g/L and lipid content is to be 27 %, resulting in 2.0 of P_C and 0.54 of P_L. At 0.03 of D (point B), dry cell weight is to be 30 g/L and lipid content is to be 38 %, resulting in 0.9 of P_C and 0.34 of P_L. From this design, higher P_L was to be obtained by higher D (point A), which was coincident with the result of continuous culture (**Figure 1** and **Figure 2**). In this fungus, therefore, operation of high D, or high growth rate, resulted in high P_L. Summing up, the maximum lipid productivity of 1.08 g/L/h was obtained in continuous culture at 100 g/L of G_I, 23.4 of C/N ratio and 0.15 h^{-1} of D, whose value was 2.9 times as great as that in batch culture (that is 0.37 g/L/h), though lipid yield in continuous culture was 2/3 of that in batch culture. The ideal system for GLA production using *Mortierella ramanniana* var. *angulispora* can be a two-stage continuous culture to separate growth phase with lipid accumulative phase. In case of oleaginous yeast (*Candida* 107), however, two-stage continuous culture was reported to have no practical advantages over a single-stage system[80].

Summary

Effect of various cultural factors (dilution rate, nutrient concentrations and C/N ratio) on lipid productivity (P_L) and lipid content were examined in a continuous culture of *Mortierella ramanniana* var. *angulispora* IFO 8187. Cultural optimization lead to the maximum P_L of 1.08 g/L/h at 100 g/L inlet glucose, 0.15 h^{-1} of dilution rate and 23.4 of C/N ratio. Under these conditions the GLA productivity was reached to more than 100 mg/L/h. The value of P_L was 3 times higher than that in a batch culture (200 g/L of initial glucose concentration, 34 of C/N ratio and 3.5 days of culture). High P_L in continuous culture may possibly be related to lower glucose concentration in the culture medium, lower C/N ratio and relatively small decrease in lipid content at a higher dilution rate or growth rate.

Section 4 Production of phospholipids containing γ-linolenic acid by *Mortierella ramanniana* grown on *n*-decane as carbon source

As for lipid class in microbial lipid production, major products were triacylglycerols. Increasing recognition of phospholipids as biologically active mediators and source of liposomes lead us to the search of microorganisms as a source of phospholipid[81]. Contents of phospholipid in microbial cells are usually lower than 5% of dry cell weight. When a methane utilizing anaerobe was used, the highest phospholipid content was reported as 17.5% of cell weight[82]. However, the growth rate of it was very slow. The author observed the content of phospholipid in the cell and γ-linolenic acid (GLA) in phospholipids increased when genus *Mortierella* grown on *n*-decane instead of glucose as shown in the sections 2, Chapter I and the section 2, Chapter II. It was also reported that higher phospholipid contents in some microbes grown on *n*-alkane than those on glucose[45,83] with their induction of intracytoplasmic membrane[84]. No extensive growth optimization of fungi using hydrocarbon as carbon source, though, has been reported.

This section deals with the investigation of the influence of cultural conditions for production of phospholipids with high GLA content of genus *Mortierella* using *n*-decane as carbon source and the separation of GLA containing phospholipids into molecular species.

Materials and Methods

Microorganisms and culture conditions. *Mortierella ramanniana* var. *angulispora* IFO 8187 was used in this study. The fungus was cultivated in the medium with *n*-decane as carbon source which combined (g/L): *n*-decane, 18.0; $(NH_2)_2CO$, 1.0; KH_2PO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 0.3; NaCl, 0.1,

malt extract, 0.2; yeast extract, 0.2; pepton, 0.1; $FeSO_4 \cdot 7H_2O$, 0.010; $CaCl_2 \cdot 2H_2O$, 0.0012; $CuSO_4 \cdot 5H_2O$, 0.00012; $ZnSO_4 \cdot 7H_2O$, 0.0010; $MnSO_4 \cdot 4H_2O$, 0.001; and Tween-80, 0.2 mL. In flask culture, 200 mL media in 500 mL baffled erlenmeyer flasks were used at 180 rpm of rotating speed and at 30℃. Culture periods was usually for 7 days. In a stirred tank fermenter, 3.0 L of media in 5.0 L fermenters (Sakuraseiki Co., model LS-5) were kept at 23℃, pH 4.0 and agitation at a speed of 400 rpm. In the fermenter culture, higher concentration of Tween-80 (1.0 mL/L) was used and 1.0 g/L of corn steep liquor and 1.0 g/L of linoleic acid were further added as a result of medium optimization. In order to prevent *n*-decane loss by aeration, *n*-decane fed continuously at 14.4 g/L/d. Aeration rate was 1.0 vvm.

Analytical Methods. Analytical methods were performed as described previously in the section 1 of Chapter I. Culture solution was taken out intermittently and dry cell weight (DC) was measured gravimetrically. Samples at steady-state condition, then, were subjected to lipid extraction and lipid analysis. Lipid was extracted from the cell, and total lipid (TL) was measured by weight. The TL was fractionated into neutral lipid (NL) and polar lipid (PL) by silicic acid column chromatography. Polar lipid fraction was further separated into glycolipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) by stepwise gradient chromatography. Glycolipids was eluted with acetone, PE was eluted with chloroform/methanol (1:1 by vol.), and PC was eluted with chloroform/methanol (1:4 by vol.), respectively. Fatty acid compositions of each lipid was analyzed by gas chromatography after methanolysis as previously described.

Samples of PE and PC were separated into molecular species on a 4.6 x 250 mm Inertsil ODS-2 column (GL Sciences Inc.) with mobile phase of acetonitrile/chloroform/methanol/water (380:150:450:20 by vol.) by HPLC

(Shimadzu, model LC-3A) equipped with refractive index detector (Showa Denko Co., model SE-61). For identification of molecular species, each retention time was compared with standard PCs and developed a graphic relationship with the theoretical carbon number (TCN) for phospholipid [69,70,85] as will be shown in **Figure 4**. Standard PCs of dilinolenoyl-PC, dilinoleoyl-PC, dioleoyl-PC , dipalmitoyl-PC, 1-palmitoyl-2-oleoyl-PC, and distearoyl-PC (Serdary Res. Lab. Inc.) were used.

Positional distribution of fatty acid in PE and PC were analyzed after the selective hydrolysis by phospholipase A_2. Five mg of PE and PC were dissolved in 5 mL of diethyl ether/methanol (98:2 by vol.) and 1 mg of phospholipase A_2 (from porcine pancreas, Sigma Co.) was dissolved in 0.5 mL of 0.1 M borate buffer (pH 7.0) including 1.6 mg potassium acetate. Reaction was started by mixing two solutions and the mixture was shaken for 2 h at room temperature. Product of hydrolysis was extracted with chloroform/methanol (1:1 by vol.). The extract was separated by TLC into free fatty acids and lysophospholipids. Fatty acid compositions of which fractions were also analyzed as mentioned above.

Results and Discussion

Medium optimization in flask culture. The effect of concentrations of nitrogen source (urea), phosphate (KH_2PO_4) and surfactant (Tween-80) on growth and lipid production were investigated (**Table 1**). Increase in urea concentration did not affect cell growth nor lipid accumulation. Under the difficulty of incorporation of hydrophobic carbon source, nitrogen source did not seem to be a limiting factor. The subsequent cultures were, then, performed at 1.0 g/L of urea. Increase in phosphate concentration up to 3.0 g/L, on the other hand, resulted in the increases in both DC and TL contents. Decrease in DC and TL values at lower concentration of

KH_2PO_4 seems to be derived from not depletion of phosphate but the lack of buffering capacity which caused increase in pH. Consequently, 3.0 g/L of KH_2PO_4 was used in the subsequent cultures except those with fatty acids (2.0 g/L).

Addition of surfactant was revealed to be indispensable for the growth on *n*-decane. Surfactant seems to help contact of cells with *n*-decane. Cell growth and TL value increased by increasing the concentration of Tween-80 up to 1.0 mL/L. This value was used for the subsequent cultures.

Table 1. Effect of nitrogen source, phosphate and surfactant on growth and lipid production

Ingredient	Content	DC (g/L)	TL (g/L)	TL/DC (%)
Urea (g/L)	0.5	2.25	0.44	19.6
	1.0	2.40	0.45	18.8
	2.0	2.42	0.43	17.8
	3.0	2.27	0.47	20.7
KH_2PO_4 (g/L)	1.0	1.42	0.26	18.3
	2.0	2.40	0.45	18.8
	3.0	3.71	0.75	20.2
	4.0	3.97	0.75	18.9
Tween-80 (mL/L)	0.0	0.57	-	-
	0.2	2.40	0.45	18.8
	0.5	2.78	0.59	21.2
	1.0	3.94	1.00	25.4
	1.5	4.02	0.94	23.4
	2.0	3.88	0.97	25.0

Flask culture, 7 d. DC: Dry cell weight
TL: Total lipid TL/DC: Lipid content

As for growth factors, corn steep liquor and several fatty acids were also investigated (**Table 2**). The values of DC and TL increased by the addition of corn steep liquor, but decreased when the added amount was higher than 1.0 g/L. Lipid content (TL/DC) was kept at about 20 % (18.3 - 21.5 %) irrespective of concentration of corn steep liquor, indicative of its little effect on lipid accumulation. Ingredients of corn steep liquor such as amino acids seemed to act as a kind of growth factor rather than as a nitrogen source. At 1.0 g/L of corn steep liquor, higher amount of yeast extract than 0.2 g/L did not increase DC value, indicating that the amount of corn steep liquor was sufficient. Concentration of 1.0 g/L corn steep liquor was added in the subsequent cultures.

Addition of free fatty acids was intended to supply an intermediate of longer carbon chain moieties than *n*-decane as well as to increase in GLA content. Effect of fatty acid addition on growth and lipid is shown in **Table 3**. No accelerating effects on growth were observed with carbon number from 10 to 12. From carbon number of 14, myristic acid, to 18s, oleic and linoleic acid, DC value was increased and reached more than 1.2-folds of control DC. Addition of linoleic acid, particularly, resulted in the highest DC, 8.05 g/L, about 2.0-folds of control DC. Linoleic acid seems to be related strongly to its growth but not to lipid accumulation. The growth accelerating effect of linoleic acid is to be made clear.

Table 2. Effect of corn steep liquor on cell growth and lipid production

Corn steep liquor content (g/L)	DC (g/L)	TL (g/L)	TL/DC (%)
(control) 0.0	5.78	0.98	17.0
0.5	6.88	1.26	18.3
1.0	8.27	1.78	21.5
2.0	8.00	1.64	20.5
3.0	5.80	1.22	21.0

Flask culture, 7d.
(Tween-80: 1.0 mL/L, KH_2PO_4: 3.0 g/L)

Table 3. Effect of additions of several fatty acids on cell growth and lipid production

Fatty acid	Content (g/L)	DC (g/L)	TL (g/L)	TL/DC (%)
(control)	0.0	3.94	1.00	25.4
Decanoic acid	0.5	3.56	0.76	21.3
Lauric acid	0.5	3.82	1.05	27.6
Myristic acid	0.5	5.26	1.27	24.2
Palmitic acid	0.5	4.28	1.33	27.7
Oleic acid	0.5	4.78	1.10	22.9
Linoleic acid	0.3	4.15	0.96	23.2
Linoleic acid	0.5	8.05	1.97	24.5
Linoleic acid	1.0	8.96	2.34	26.1

Flask culture, 7 d.
(Tween-80: 1.0 mL/L, KH_2PO_4: 3.0 g/L)

Table 4. Time course of lipid content and distribution in the culture with and without linoleic acid (LA)

Culture period (d)	Control TL/DC (%)	Lipid distribution wt% in DC TG	DG	FFA	FS	PC	PE	LA addition TL/DC (%)	Lipid distribution wt% in DC TG	DG	FFA	FS	PC	PE
5	13.1	6.0	0.1	0.3	1.6	3.0	2.1	17.8	5.6	0.1	5.1	2.7	2.5	1.8
7	17.3	9.8	0.2	0.3	2.9	2.7	1.4	22.5	12.7	0.5	1.3	4.0	2.6	1.4
10	25.3	17.3	0.4	0.1	3.8	2.5	1.2	26.6	17.5	0.5	0.1	4.4	2.8	1.3

TL: Total lipid DC: Dry cell TG: Triacylglycerol DG: Diacylglycerol FFA: Free fatty acid FS: Free sterol PC: Phosphatidylcholine PE: Phosphatidylethanolamine

Lipid content of flask culture with linoleic acid addition was compared with that of control at three culture periods (**Table 4**). Values of TL/DC

increased according to the increase in culture period. At 7 days, TL/DC with linoleic acid addition was 30% higher than that of the control but resulted in the similar values at 10 days. Increase in TL derived mainly from triacylglycerol, or stored lipid. With linoleic acid addition, a remarkably high value of free fatty acid (5.1%) was analyzed at 5 days, which seemed to be related to the added linoleic acid. The content of free fatty acid was rapidly decreased and no difference was observed at 10 days indicative of assimilation of linoleic acid by the cell. Value of PL/DC on *n*-decane was usually higher than that on glucose, but there was little difference in PL (mainly PC and PE) between linoleic acid addition and the control.

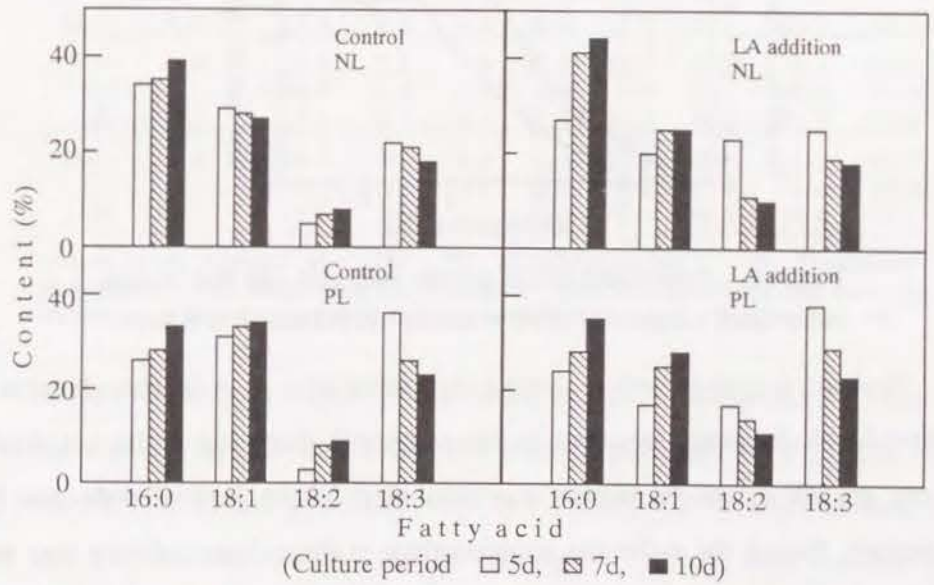


Figure 1. Fatty acid composition of neutral lipid (NL) and polar lipid (PL) fraction in control and linoleic acid (LA) added flask culture.

Time course of fatty acid composition in NL and PL is shown in **Figure 1**. At 5 days, linoleic acid contents in both fractions increased (6.0-fold in PL and 4.0-fold in NL) by the addition of linoleic acid. But fatty acid at 10 days resulted in similar profiles. Content of GLA in PL increased by the

addition of linoleic acid at 5 and 7 days owing to higher substrate (linoleic acid) content of Δ6-desaturase to GLA. In NL fraction, no differences in GLA content were observed except at 5 days, and early stage of growth. Addition of linoleic acid was not, consequently, effective for increase in GLA content.

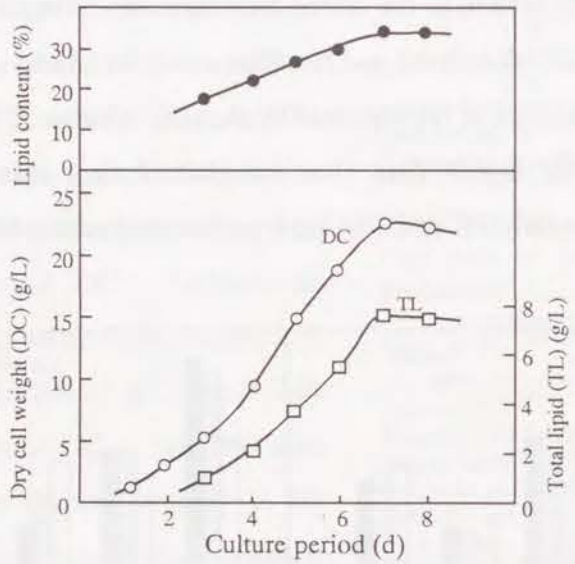


Figure 2. Time course of cell growth, total lipid and lipid content in fed-batch culture. 14 g/L/d *n*-decane was fed from 3 to 8 days.

Fed-batch culture with a stirred-tank fermenter. A batch culture in a stirred-tank fermenter resulted in lower growth than that in flask culture. Low growth in the fermenter was attributed to the loss of *n*-decane by aeration, though the *n*-decane concentration in the culture solution was not determined. To prevent the loss of *n*-decane, *n*-decane was continuously pumped into the culture broth at 14.4 g/L/d, whose operation was called fed-batch culture. Moreover operational temperature was lowered to 23℃ to decrease the evaporation loss of *n*-decane. Time course of the culture in fermenter is shown in **Figure 2**. At seven days, 22 g/L of DC and 8 g/L of TL were observed and those values were almost 3 and 4-fold higher than

those obtained in the flask culture, respectively. Higher DC in the stirred-tank fermenter is likely to be attributed to the agitation and pH control. Agitation together with high concentration of Tween-80 must facilitate contact between cells and *n*-decane. The fed-batch operation of *n*-decane made it possible to keep relatively high concentration of *n*-decane at late logarithmic growth phase or stationery growth phase when lipid was accumulated without cell division, which should result in high TL/DC.

Table 5. Lipid content and their fatty acid compositions with positional distribution of fatty acids in PC and PE

Lipid class	Content/ DC (%)	Fatty acid composition (%) 16:0	16:1	18:0	18:1	18:2	18:3*	UFA (%)	DU
NL	34.3	40.4	4.5	3.2	23.8	8.4	14.8	51.9	89.9
GL	0.7	32.6	3.0	5.3	40.6	10.7	10.3	65.0	96.3
PC	2.4	23.0	3.0	1.6	12.2	23.7	31.2	70.4	156.5
c1		45.6	1.4	5.2	8.7	9.1	21.1	40.3	91.6
c2		6.5	3.7	0.5	16.5	37.4	35.2	92.8	200.6
PE	1.3	30.1	3.7	0.9	18.8	13.8	28.8	65.3	136.7
c1		49.1	1.8	4.5	10.8	6.0	17.0	35.6	75.6
c2		10.1	3.5	0.1	32.0	19.4	34.5	89.4	177.8

Culture in the fermenter at 7 days PC: Phosphatidylcholine PE: Phosphatidyl-ethanolamine NL: Neutral lipid GL: Glycolipids UFA: Total unsaturated fatty acids (%) DU: Degree of unsaturation (number of double bonds/100 molecules) * γ-Linolenic acid

Table 5 summarizes lipid profile of the culture in fermenter at 7 days. The ratio of PL to DC was 5.1% and major lipid class of PL were PC (2.4%), PE (1.3%) and glicolipids (0.7%). Nearly 1/3 of GLA existed in PC and PE, whereas about 10% in glicolipids. Oleic acid content in glicolipids was much higher than those in PC and PE. Degree of unsaturation was higher in PC than that in PE due to high content of linoleic acid. Linoleic acid was concentrated at position 2, three times more than position 1, while saturated acids (16:0, 18:0) were concentrated at position 1. Monoenoic acids (18:1, 16:1) were also higher at position 2, though degree of positional specificity of monoenoic acid was not so strong as those of

saturated acids and linoleic acid. Distribution of GLA was also inclined to position 2, which was not so evident compared with linoleic acid.

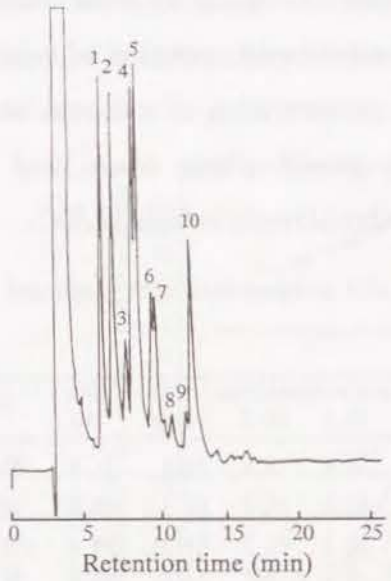


Figure 3. HPLC chromatogram of molecular species of phosphatidylcholine. Column, Inertsil ODS-2 (4.6 mm × 250 mm); and solvent system, acetonitrile/ chloroform/methanol/water (380 : 150 : 450 : 20) at a flow rate of 1.0 mL/min. Peaks were identified as, 1, 18:3-18:3; 2, 18:3-18:2; 3, 18:2-18:2; 4, 18:3-18:1; 5, 18:3-16:0; 6, 18:2-18:1; 7, 18:2-16:0; 8, 18:3-18:0; 9, 18:1-18:1; and 10, 18:1-16:0, the order of designation does not indicate the separation of positional isomers.

Phospholipid separation into molecular species. Molecular species of each PC and PE fraction was further analyzed with HPLC. **Figure 3** shows HPLC chromatogram of PC fraction. Each peak was assigned using the concept of TCN for triacylglycerol as shown in **Figure 4**. From the standard PCs to 18:3-18:3-PC and fatty acid composition of the PC sample, each peak in **Figure 3** was assigned as shown in **Figure 3**. A good linear relationship between TCN and log(RRT) indicates the validity of the assignment. The values of RRT for both PC and PE were identical. **Table 6** summarizes the content of molecular species of PC and PE. Main species were 18:3-16:0, 18:3-18:2, 18:3-18:3, 18:1-16:0, and 18:3-18:1. As the cell growth, content of oleic acid increased but that of GLA decreased in PC molecules. Contents of 18:3-18:3 and 18:3-16:0 were higher in culture on *n*-decane than that on glucose, while those of 18:3-18:2 and 18:3-18:1 were lower. Contents of molecular species including GLA were more than

60% of PC class and 46% of PE. Productivities of PC and PE containing GLA were estimated to be about 300 and 125 mg/L, respectively.

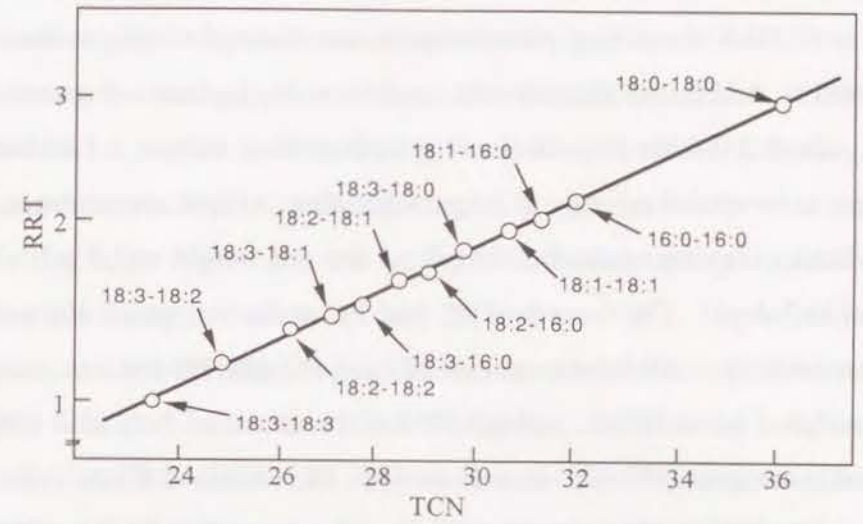


Figure 4. Theoretical carbon numbers (TCN) of phosphatidylcholines and phosphatidilethanolamines vs. their relative retention times (RRT).
RRT = Retention time of each PC molecule/(retention time of 18:3/18:3-PC)
TCN = (TC-2 x DB) - ΣUi
where TC: total carbon number, DB: number of double bond,
Ui = (Oleate: 0.65, linoleate: 0.85, linolenate:0.2)

Table 6. Molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in several culture conditions

Molecular species (%)	PC (3d)	PC (7d)	PE (7d)	PC (grown on glucose)
18:3-18:3	17.2	11.7	6.5	6.8
18:3-18:2	16.8	16.2	5.4	20.5
18:3-18:1	3.0	12.0	11.4	16.1
18:3-18:0	2.3	2.3	-	-
18:3-16:0	29.3	18.8	22.6	15.0
18:2-18:2	6.2	4.1	17.6	3.5
18:2-18:1	1.4	5.9	4.0	9.5
18:2-16:0	6.8	7.4	5.6	9.0
18:1-18:1	-	1.7	-	9.6
18:1-16:0	10.6	13.6	22.6	10.0
GLA-PL*	68.6	61.0	45.9	58.4

* Total percentage of molecular species containing γ-linolenic acid (%)

Summary

The influence of cultural conditions on production of GLA containing phospholipids of genus *Mortierella* using *n*-decane as carbon source and the separation of GLA containing phospholipids into molecular species were investigated. Addition of linoleic acid resulted in the highest cell growth, 8.05 g/L, about 2.0-folds of control cell growth in flask culture. Linoleic acid seems to be related strongly to its growth but not to lipid accumulation. The fed-batch culturing resulted in 22 g/L of dry cell weight and 8 g/L of total lipid at 7 days. The content of PC and PE in the cell was 2.4% and 1.3%, respectively. Molecular species of each PC and PE fraction was further analyzed by an HPLC. About 30% of the esterified fatty acid was GLA, and more than 60% PC as well as 46% PE contained GLA. The productivities of PC and PE containing GLA were estimated to be about 300 and 125 mg/L-medium, respectively.

CONCLUSION

In this thesis, the author described the production of lipids and GLA containing lipids by cultures of *Mortierella* fungi. *M. isabellina* and *M. ramanniana* var. *angulispora* were selected, and the effects of the cultural conditions, especially the carbon source, nitrogen source, C/N ratio, culture temperature and pH, on the lipid and GLA productivities were examined. Culture at a higher glucose concentration than 100 g/L resulted in an increase in lipid content as well as cell growth. The maximum lipid productivity of 0.69 g/L/h in a batch culture was obtained by *M. isabellina* IFO 7884. Five strains of the genus *Mortierella*, including *M. isabellina* IFO 7884 and *M. ramanniana* var. *angulispora* IFO 8187, attained GLA yields exceeding 2 g/L-medium. In the operational modes of repeated batch culture and continuous culture, both lipid productivity and GLA productivity increased in comparison with those in a batch culture. In a continuous culture of *M. ramanniana* var. *angulispora* IFO 8187, a lipid productivity of 1.1 g/L/h and a GLA productivity of 106 mg/L/h were obtained. The value of lipid productivity was 3 times higher than that in a batch culture. Furthermore, the production of phospholipids containing GLA was examined using *n*-decane as a carbon source. In a *n*-decane culture, higher polar lipid content and higher GLA content in the lipid were observed. The results in each chapter can be summarized as follows.

CHAPTER I

In the screening of fungi which produce GLA as well as lipid, *Mortierella isabelina* was found to accumulate a large amount of lipid intracellularly among the members of the order Mucorales. The content of GLA of *M. ramanniana* var. *angulispora* IFO 8187 was relatively high

among the members of the genus *Mortierella* under the cultural conditions with either glucose or *n*-decane as the carbon source.

CHAPTER II

In a flask culture, the highest lipid content, more than 80% of dry cell weight, was obtained at high C/N ratio of 343 with glucose as the carbon source. The maximum production of lipid, on the other hand, occurred at a lower C/N ratio of 11.4. In a culture at low pH, lipid production increased, but cell growth decreased. Culture on *n*-decane gave lower lipid content than that of glucose culture with high C/N ratio. A culture on *n*-decane, however, gave a higher polar lipid content and higher GLA content in the lipid compared to the culture on glucose.

Furthermore, in order to increase the lipid productivity, a culture with a high concentration of the carbon source was performed. *M. isabellina* IFO 7884 was able to grow at glucose concentrations higher than 200 g/L without decrease of growth rate. The maximum dry cell weight and lipid production of 156 and 83 g/L, respectively, were obtained in a culture with 390 g/L glucose. The highest lipid productivity, 0.69 g/L/h, was obtained in a culture with the lower glucose concentration of 270 g/L.

CHAPTER III

Five strains, including *M. isabellina* IFO 7884 and *M. ramanniana* var. *angulispora* IFO 8187, attained GLA yields exceeding 2 g/L-medium in high glucose concentration medium. The culture conditions with a C/N ratio of 34 gave the highest lipid production and GLA yield by *M. ramanniana* var. *angulispora* IFO 8187. Lipid productivity and GLA productivity increased in repeated batch cultures and continuous cultures. In a continuous culture of *M. ramanniana* var. *angulispora* IFO 8187, a lipid productivity of 1.1 g/L/h and a GLA productivity of 106 mg/L/h were

obtained. The solvent winterization process was effective to increase in GLA concentration in the fungal oil, resulting in 8.3% of GLA from the original oil at 5.7% GLA, with acetone as the solvent. Furthermore, the culture conditions to obtain phospholipids containing GLA were clarified using *n*-decane as a carbon source. The productivities of GLA-containing species of PC and PE were estimated to be about 300 and 125 mg/L, respectively, in a *n*-decane culture.

Lately, microbial lipid production, especially PUFA production, has become of general interest. In Japan, the major sources of fats and oils are dependent on those from other countries. Microbial lipid production is important not only as a method of production of fatty acids difficult to obtain from plant or animal oils, but also in terms of national security and global resource preservation. The industrial production of GLA-containing oil from the genus *Mortierella* has been already begun in Japan, as the first "Single Cell Oil". The GLA-containing oil is used as a food additive, as a health food in the form of drinks and tablets, and mixed in cosmetic to keep the skin moist. The reasons why microbial GLA production by the *Mortierella* fungus was industrialized first in the world lies in low occurrence of GLA in conventional plant resources and the realization of extremely high productivities of lipids and GLA.

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